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Growth, 1952, **16**, 59-63.

ACID AND ALCOHOL TOLERANCE IMPARTED BY THIAMIN

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Previous experiments (GROWTH, 15, 267-287 (1951)) had shown that thiamin (vitamin B_1) makes the yeast variety "Old Process" tolerant against alcohol. With 7.5% alcohol, the yeast died in a good medium if this did not contain thiamin, but with thiamin, the yeast multiplied in spite of the 7.5% alcohol. The same vitamin is also necessary to protect *Lactobacillus fermenti* against lactic acid. The critical acid concentration is 0.15%. The amount of 0.2 ppm. thiamin enables the bacterium to grow in spite of the acid.

The double action of thiamin suggested that the protective effect might apply to both species and both compounds, i.e., the lactobacillus might be protected against alcohol as well as acid, and the yeast might have become tolerant to acid as well as alcohol.

In the case of the yeast, not much effect could be hoped for, since all yeasts are very tolerant to hydrogen ions, but the test with the lactobacillus should be convincing.

As the figure shows, the lactobacillus had become quite alcohol-tolerant by growing in a medium containing 0.2 ppm. thiamin. The cloudiness of the cultures with thiamin indicates good growth of the lactobacillus even in the presence of 3% alcohol which was sufficient to prevent growth when no vitamin was present.

To make these differences more quantitative, the bacteria were counted after four days of growth at 37° C., the medium being a synthetic mineral-glucose-ammonia solution to which 1 g. of tryptose and 4.25% of thiamin assay medium (Difco) per liter had been added. The thiamin content of the cultures containing vitamin was 0.2 ppm.

* This work was done in the Bacteriological Laboratory of the University of Nebraska, while there as a guest of the university, and with the financial support by a grant from the Federal Security Agency, Public Health Service, Bethesda, Md.

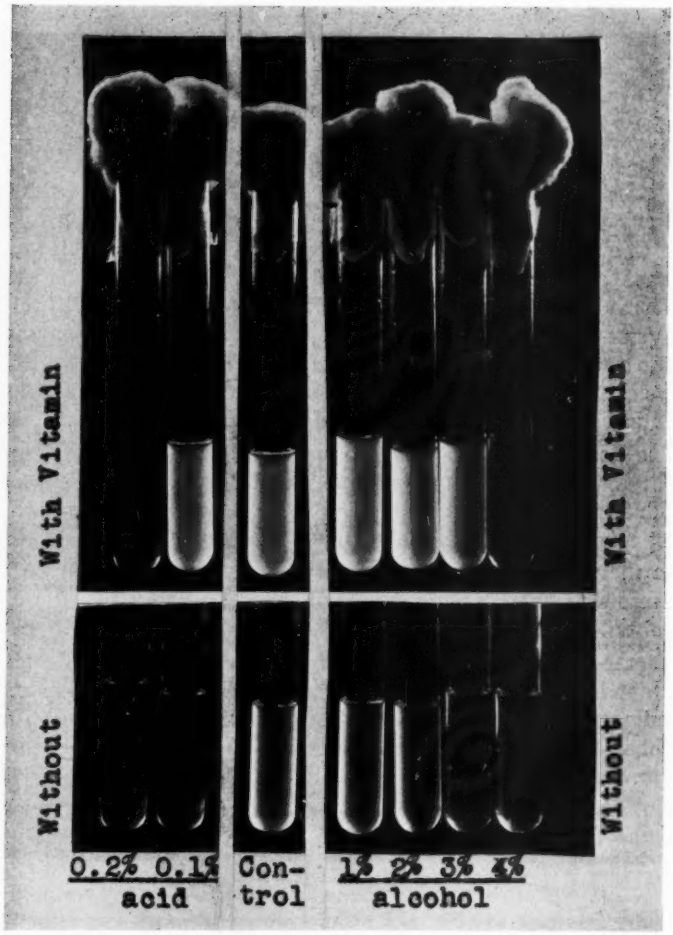

FIGURE 1
INCREASE OF TOLERANCE OF *Lactobacillus fermenti* TOWARDS LACTIC ACID AS WELL AS ALCOHOL, BY THIAMIN (VITAMIN B_1)

TABLE 1
INCREASE OF TOLERANCE TO ACID AS WELL AS ALCOHOL PRODUCED BY THIAMINE IN *Lactobacillus fermenti*

Cells of the Lactobacillus after 3 Days' Growth

		With vitamin	Without vitamin
Lactic acid added	0.1%	171,500,000	120,000
	0.2%	no growth	no growth
Alcohol added	0%	357,000,000	80,000,000
	1%	288,500,000	49,650,000
	2%	352,500,000	———
	3%	384,500,000	7,390,000
	4%	360,000	18,500

Figures as well as plate counts prove that thiamin protects the lactobacillus as well as the yeast against the injurious effect of alcohol. The tolerance imparted by the vitamin is therefore not specific for the metabolic products of the species under test. Attention may be called here to the observation that thiamin has been found to decrease the toxic effect of alcohol in humans where alcohol is not a metabolic product. The fact that in alcoholic as well as in lactic fermentation, glucose-phosphate and pyruvic acid are intermediate products, has probably nothing to do with the increased tolerance. It seems more likely that the apo-enzyme (or the protoplasm) becomes generally less vulnerable by combining with the vitamin. The organisms had multiplied in the presence of the vitamin, and had thus been able to incorporate it into their apo-enzyme or protoplasm.

Since the incorporation of vitamin into the protoplasm of a cell may increase its tolerance not only to its own metabolic products, but to other harmful compounds as well, vitamins may have a more important place in physiology than has been commonly believed.

The above-mentioned protection of a cell against a foreign product (alcohol) suggests that vitamins may be of pharmaceutical importance. A good example is the effect of Vitamin C on injured cells of *Streptococcus lactis*.[1]

> "Vigorous fresh cells were not benefited by ascorbic acid, 0.02%; in fact, there was a decrease in some cases. When the cells were injured by heating, by holding at 0° for several days, or by the use of water in place of buffer for washing to remove medium and metabolic products from the bacteria, the power of

[1] RAHN AND HEGARTY. 1938. *Proc. Soc. Exp. Biol. & Med.* **38**, 218.

the cells to produce colonies on agar was lost more rapidly than their fermenting capacity. The same had been observed earlier with yeast cells. In all these cases, the cells were benefited by ascorbic acid. In three experiments, the centrifuge became so overheated that the cells failed altogether to produce colonies though they still fermented. Here, the ascorbic acid caused the greatest increase, namely 400%, 500%, and 1100%. Different kinds of injury all resulted in a need for ascorbic acid, and it seems that the greater the injury, the greater is the benefit from this compound, relatively as well as absolutely."

The prerequisite for such effect is probably the chemical combination of the vitamin with the cell protoplasm. It has been shown by Cohen and Rahn[2] that growing bacteria remove vitamin rapidly from a nutrient medium and incorporate it in their cells in a form available for other microorganisms. This results in a concentration of the vitamin from the very dilute solution of the medium into the cells of bacteria. As the cells of *E. coli* grown in 1 liter of liquid medium have a dry weight of approximately 220 mg.,[3] the vitamin content of 1 liter of liquid may thus be concentrated biologically into a mass of cells weighing less than 1 gram. Animals feeding on bacteria may increase the degree of concentration still more if they digest the bacterial protein and store the vitamin. The vitamin content of cod liver oil may originate in this way.

It is imaginable that other vitamins and perhaps other substances may be incorporated into the cell protoplasm and thereby protect it against cell products.

The author and his wife have taken vitamin pills regularly for a large number of years, and have been remarkably free from colds in a coughing and sneezing world. There may be no causal relation between vitamins and freedom from colds, but a statistical study of this possibility seems worthwhile.

There is no special atomic structure in the various vitamins that makes them seem to be especially predestined for the purpose of increasing tolerance to metabolic products, but their incorporation into the protoplasm of growing cells has been proved by Cohen and Rahn, at least for one vitamin.

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[2] COHEN AND RAHN. 1951. GROWTH **15**, 141.

[3] RAHN. 1932. Physiology of Bacteria. Philadelphia, Blakiston, p. 397.

Summary

The yeast Old Process, when grown in a medium containing thiamin, becomes more tolerant to its normal metabolic product which is alcohol. *Lactobacillus casei,* when grown in a medium containing thiamin, becomes more tolerant to its normal metabolic product which is lactic acid, but it also becomes more tolerant to alcohol which is *not* its metabolic product. The protection imparted by the incorporation of the vitamin into the protoplasm does not depend upon the specific metabolism of the protected species.

Growth, 1952, **16**, 65-74.

CONDITIONING OF THE HOST CELL AS A FACTOR IN THE TRANSFORMATION PROCESS IN CROWN GALL

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(Received for publication April 1, 1952)

The crown-gall bacterium possesses the ability to transform irreversibly normal plant cells to tumor cells in short periods of time. When the cellular alteration has been fully accomplished the continued abnormal proliferation of the altered cells becomes entirely independent of the inciting bacteria. This indicates that a factor of considerable biological interest passes from the bacteria to the host cells and brings about a complete and permanent change in the subsequent behavior of the affected cells. The transformation of normal cells to tumor cells appears, moreover, to take place gradually (Braun, 1943, 1951), leading in a three to four-day period to an autonomous, rapidly growing type of cell. Cells altered in 34 hours, on the other hand, develop very slowly and the resulting tumors become encapsulated, reaching moderate size only after prolonged incubation. There is, furthermore, a lag of somewhat more than one day between the introduction of the bacteria into the host and the first evidence that the cellular alteration has occurred (Braun, 1943, 1947; Braun and Mandle, 1948). This lag period may represent either an incubation period necessary for the bacteria to establish themselves in the host and to elaborate the tumor-inducing principle in amounts sufficient to bring about the cellular alteration, or it may represent a period of conditioning necessary to make the host cells susceptible to transformation. It is with an attempt to distinguish between these two possibilities that the present paper is concerned.

EXPERIMENTAL METHODS

Earlier studies (Braun, 1947; Braun and Mandle, 1948) demonstrated that normal plant cells may be converted into tumor cells by the crown-gall bacteria at a temperature of 25° C. but not at 32° C.

Once the cellular transformation was accomplished at 25° C., however, the tumor cells developed into neoplastic overgrowths equally well at both temperatures. With the use of this method the process of transformation could be stopped abruptly and completely at any desired time by simply placing and holding the inoculated plants at 32° C. The size and rate of growth of the tumors that developed at 32° C. reflected the degree of cellular alteration that had taken place at the lower temperature up to the time the plants were placed and held at 32° C. This method formed the basis of the experimental work reported here.

Kalanchoe daigremontiana Hamet and Perrier was used as the test plant. Plants grown in 2.5-inch pots were used when they reached a height of about 3 inches.

The virulent B6 strain of *Agrobacterium tumefaciens* (Smith and Town.) Conn was used as the test organism. A moderately heavy bacterial suspension of a 24 or 48-hour agar culture made up in dilute nutrient dextrose broth was used as the source of inoculum.

A thermostatically controlled glass chamber in which the temperature was maintained at 24.5° - 25.0° C. was used for experiments carried out at that temperature. A constant-temperature room maintained at 32° - 33° C. was used for studies involving that temperature. In both instances the relative humidity was held at between 80 and 90 per cent. Plants held at both 25° C. and 32° C. were kept under constant illumination by means of fluorescent lamps. All results were recorded about 16 days after the plants had been placed at 32° C. for the final incubation at that temperature.

Experimental Results

A wound appears to be essential if tumors are to form in crown gall. Juices liberated from the wounded cells diffuse a distance of several millimeters from the site of the wound and stimulate division of the resting cells that are in contact with those juices. The wound is usually healed within a week to ten days, after which time the cells surrounding the injury again become resting cells.

It appeared possible to study the effects of wounding on the transformation process by making a fresh wound into healing tissue and permitting the bacteria to act on the newly exposed cells for 24 hours, a period not in itself sufficient to permit the cellular transformation to occur in cells not previously under the influence of a wound. Earlier

studies (Braun and Mandle, 1948) had indicated that tumors of the largest, most rapidly growing type are initiated in *Kalanchoe* plants when the host cells are altered between the second and third days after inoculation. A comparison of the response to the effects of the tumor-inducing principle on cells that had been under the influence of a wound for 48 hours before inoculation and cells that had never been exposed to wounded tissue was therefore made.

In these experiments *Kalanchoe* plants about 3 inches tall were used. The plants were punctured through internodes at several points along the stem with a sterile dissecting needle. To prevent drying, the freshly wounded areas were loosely covered with a rubber tape known as "Sterilastic"; and the plants were permitted to remain on a greenhouse bench for 48 hours. Following the 48-hour period a moderately heavy suspension of the crown-gall bacteria was introduced into the wounded areas by means of a dissecting needle that had a slightly larger diameter than did the needle employed for making the original sterile punctures.

A second set of *Kalanchoe* plants comparable in every respect with those used in tests described above was similarly treated except that the bacteria were inoculated directly into plant tissues that had not previously been under the influence of a wound. Immediately after inoculation both sets of plants were placed for 24 hours in a chamber the temperature of which was controlled at 25° C. Following the incubation at 25° C., the plants were placed and held for 16 days at 32° C. The higher temperature stopped the transformation process and permitted the development of tumors from cells altered during the 24-hour incubation at 25° C.

The results obtained in experiments of this type were striking and entirely reproducible (Figure 1). That set of plants that had been inoculated with bacteria in tissue not previously under the influence of a wound showed almost without exception no reaction at the points of inoculation. The other group of plants that had been wounded 48 hours prior to inoculation with the bacteria developed moderately fast growing tumors at all of the points of inoculation. The tumors developed early. Three days after the plants had been placed at 32° C. overgrowths were observed in the inoculated areas. Tumors initiated by the freshly applied bacteria in 24

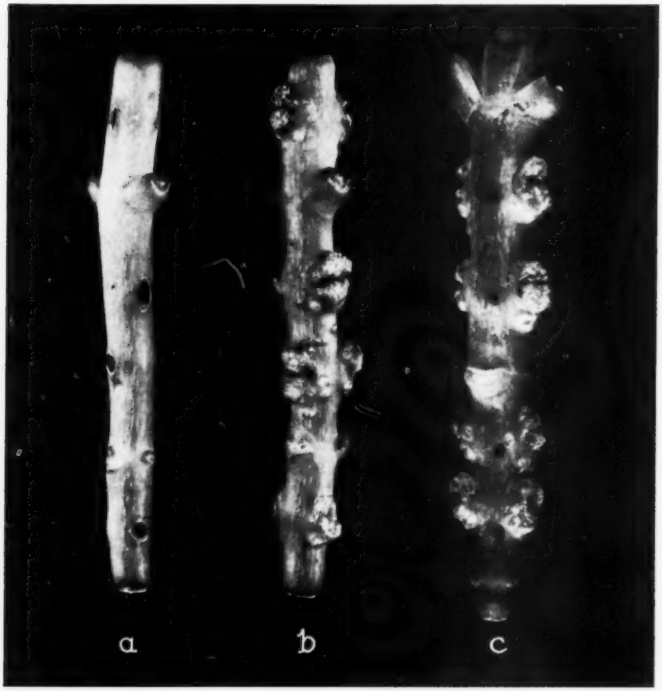

FIGURE 1

Crown-gall bacteria were inoculated (a) into tissues that had not previously been under the influence of a wound and (b) into tissues that had healed for 48 hours prior to inoculation. In both instances the bacteria were permitted to act for 24 hours at 25° C., after which time the plants were placed and held at 32° C. for 16 days. (c) shows the two types of response on the same stem segment.

hours at 25° C. following a 48-hour healing period were only slightly smaller than were tumors obtained in a similar period when the bacteria were permitted to multiply and establish themselves fully in the host for 48 hours at 32° C. prior to the incubation of the host-parasite complex at 25° C. for 24 hours. These results indicate (*1*) that the conditioning process takes place at both 25° C. and 32° C. and (*2*) that the bacteria require only a very short incubation period following their introduction into a host in order to accomplish the cellular

transformation provided the host cells acted upon by the bacteria are adequately conditioned.

Since these experiments demonstrated that a period of activation of the host cells resulting from the stimulus of a wound was essential if the cellular transformation was to occur in crown gall, an attempt was next made to study the development of this conditioning process in the host cells. In these experiments sets of plants were punctured through internodes with a sterile needle at 24-hour intervals for a period of five days. Additional plants were wounded at such intervals as to permit 0, 3, 6, 12, and 18-hour healing periods to take place before inoculation with the bacteria. On the sixth day all of the sterile punctures were inoculated by needle puncture with a suspension of the bacteria and placed at 25° C. for 24 hours. The results of these studies, representative stem segments of which are pictured in Figure 2, demonstrate that the process of conditioning takes place

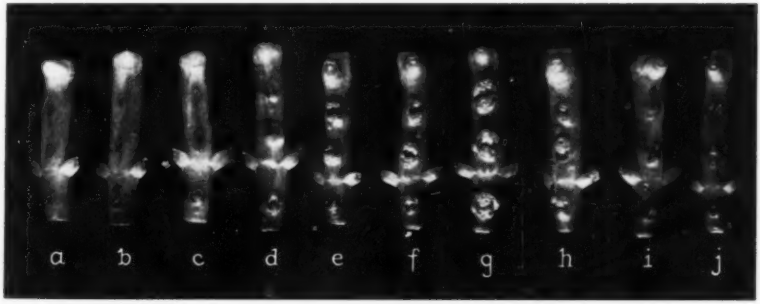

FIGURE 2

Kalanchoe stems were wounded with a sterile dissecting needle at three points. The sterile wounds were permitted to heal for (a) 0, (b) 3, (c) 6, (d) 12, (e) 18, (f) 24, (g) 48, (h) 72, (i) 96, (j) 120 hours before being inoculated into the wounded areas with crown-gall bacteria. The bacteria were allowed to act for only 24 hours at 25° C., after which time the plants were placed and held at 32° C. Note that the maximum response was obtained when cells in the wounded areas were permitted to heal for 48 hours prior to inoculation.

gradually. When plants were permitted to heal for only three hours before inoculation with the bacteria, no reaction was found in the inoculated areas after a suitable incubation period. A 6- and 12-hour healing period prior to bacterial inoculation permitted the development of small overgrowths at the points of inoculation. As the healing

period increased from 6 to 48 hours prior to inoculation with the bacteria, the size of the resulting tumors also increased. A maximum degree of transformation was achieved between the second and third days after wounding. When, on the other hand, a sterile wound was permitted to heal for four or five days before inoculation with the inciting bacteria, small tumors or no tumors at all resulted at the points of inoculation. The temperature and relative humidity under which normal wound healing occurs determines to some extent the period of time that the cells remain susceptible to transformation.

These experiments indicated that wound healing plays an essential rôle in the process of transformation of normal cells to tumor cells in crown gall. It appears, therefore, that conditioning of the host cells may represent either (*1*) a period of activation necessary to make the cells susceptible to transformation, or (*2*) a healing period necessary to create an environment suitable for the bacteria to elaborate the tumor-inducing principle.

In order to test this second possibility, an attempt was made to establish conditions in freshly wounded tissues approximating as closely as possible those that exist in healing tissues. In these experiments *Kalanchoe* plants were cut through an internode at about the middle of the plants. The lower halves of the plants were discarded. The freshly cut surfaces of the top halves of the plants were covered with "Sterilastic" tape and placed in an upright position under a moist bell jar for 48 hours to permit healing to take place. At the end of that period the conditioned cells of the cut surfaces of the top halves of plants were placed and held firmly in intimate contact with freshly cut, inoculated stem surfaces of lower halves of plants that had not previously been under the influence of wounded tissue. In these experiments, as well as in those of a similar type described below, the plants were placed under a moist bell jar for 21 hours at 25° C. Following incubation at 25° C., the two halves of the plants were again separated, the top portions being discarded while the lower halves of the plants were placed at 32° C. for 16 days. Tumors were not initiated on the cut surfaces of the lower halves of the plants in experiments of this type. In order to determine whether tumors would be initiated under similar conditions in healing cells, tests were carried out in which the cut stem surfaces of both top and bottom halves of plants were permitted to heal for 48 hours before being inoculated and brought into close contact with each other for 21 hours

at 25° C. In these experiments moderately fast growing tumors were produced on the inoculated surfaces of the lower halves of the plants. In still other experiments freshly cut stem surfaces of top halves of plants were inoculated with crown-gall bacteria. These were permitted to heal for 48 hours before being placed in close contact with freshly cut stem surfaces of lower halves of plants that had not previously been under the influence of a wound. Tumors were not produced on the cut stem surfaces of the lower halves of the plants. Finally, wounded surfaces of lower halves of plants were permitted to heal for 48 hours before being brought into contact with freshly wounded inoculated cut stem surfaces of upper halves of plants. In experiments of this type tumors were produced on the cut stem surfaces of the lower halves of the plants. The results of these experiments demonstrated that tumors were not initiated in 21 hours at 25° C. on freshly cut, inoculated stem surfaces of the lower halves of the plants in the two tests in which freshly exposed cells not previously under the influence of a wound were used. In the two experiments in which the cut stem surfaces of the lower halves of the plants had been permitted to heal for 48 hours before being brought into contact with inoculated upper halves of plants tumors of moderate size were produced on the cut stem surfaces. It is clear, therefore, that although healing cells were in intimate contact with cells of a freshly cut surface, the presence of these conditioned cells did not facilitate transformation by the bacteria of the newly exposed cells. It is equally clear that cells conditioned for 48 hours were capable of being readily transformed to tumor cells by the bacteria in the presence of freshly wounded tissue. It seems probable, therefore, that conditioning is concerned with an activation of the cell itself rather than with environmental conditions affecting the capacity of the bacteria to elaborate the tumor-inducing principle.

Since conditioning of the host cells was shown to take place gradually, it seemed possible that some normal cellular constituent might be elaborated in host cells in progressively increasing amounts during the activation of the cells following wounding. An attempt was therefore made to learn whether wounded normal tissues, when ground up, mixed with bacteria and inoculated into normal tissues not previously under the influence of a wound, would permit the initiation of tumors in 24 hours at 25° C. In these experiments about one hundred *Kalanchoe* plants were cut through an internode with a sharp scalpel

and the freshly cut surfaces were covered with "Sterilastic" tape to prevent drying. The plants were permitted to remain on a greenhouse bench for 48 hours. Following the healing period a slice of tissue 1-to-2 millimeters in thickness was removed from immediately below the original wound in each plant. The cells in this region had, of course, been activated by the cell juices resulting from the original wound. The tissue slices were thoroughly ground in a mortar and the plant juice obtained was centrifuged at about 2500 r.p.m. for several minutes to remove the large particles. Bacteria taken directly from an agar slant were added to the supernatant fluid and the resulting suspension was inoculated into *Kalanchoe* tissues that had not previously been under the influence of a wound. These experiments demonstrated that extracts of activated tissue when mixed with bacteria did not permit the cellular alteration to occur in 24 hours at 25° C. This may mean either (*1*) that a normal cellular component concerned with conditioning is elaborated by the host cells during the early stages of the healing process but that this factor is effective only if present in the intact cell, possibly by virtue of the fact that it activates or is activated by the tumor-inducing principle, or (*2*) that conditioning may represent an alteration of the surface of the cells enabling the tumor-inducing principle to penetrate the cells.

Discussion

It has long been recognized that a wound is necessary if tumors are to form in crown gall. The reason for this has remained obscure. The findings reported here demonstrate that a conditioning of the host cells which results from the stimulus of a wound is necessary if the cellular transformation is to occur. The tissues around wounds that had been permitted to heal for 48 hours before inoculation with crown-gall bacteria responded vigorously with tumor formation when the inciting organism acted on these healing cells for only 24 hours at 25° C. When, on the other hand, tissues not previously under the influence of a wound were similarly inoculated with the bacteria, tumors were not initiated in 24 hours at 25° C.

Previous studies (Braun and Mandle, 1948) indicated that it is possible to correlate the degree of alteration of tumor cells, as measured by the rate of growth of the resulting tumors, with the period in the wound-healing cycle in which the alteration occurs. Very small,

slowly growing tumors are initiated, for example, when cells are altered either in the first 34 hours or subsequent to 90 hours after the bacteria are inoculated into a host. Moderately fast growing tumors are produced when the cellular alteration occurs during the first 44 hours or subsequent to the 72nd hour after the wound is made. A minimum period of about 60 hours appears to be required for the initiation of large rapidly growing tumors. Tumors are not initiated in the first 24 hours or, for the most part, subsequent to the fifth day after the wound is made. These results can be accounted for entirely on the basis of conditioning of the host cells. This conditioning process which renders the cells susceptible to transformation takes place independently of the bacteria at temperatures of both 25° C. and 32° C., although the cellular alteration occurs only at the lower temperature.

The conditioning effect may involve permeability changes in the cell membranes which enable the penetration of the tumor-inducing principle into conditioned cells. These results are also understandable, on the basis of work reported here, if it is assumed that a normal cellular component of as yet unknown nature is elaborated in gradually increasing amounts during the early stages of the healing process, reaching a maximum concentration between the second and third days after the wound is made. At that time the normal host cells in the region of the wound begin to divide and, since this normal component is assumed to be no longer elaborated after normal cell division begins, its concentration would decrease in proportion to the number of divisions that the cells underwent during the healing of the wound. The degree of cellular alteration reached at any given time in the normal wound-healing cycle would, according to this concept, be dependent not only on the presence of the tumor-inducing principle but on the concentration in the activated host cells of this normal factor as well. It has been demonstrated moreover that if the host cells are not adequately conditioned, as appears to be the case in both the early and late stages of wound healing as well as in most normal cells not under the influence of a wound, then the cellular alteration will not occur despite the presence of the tumor-inducing principle.

Exploratory studies have shown that the period following wounding necessary to condition the host cells for transformation, as well as the period in which those cells remain susceptible to transformation, may vary considerably from one plant species to another.

Summary

Conditioning of the host cells which results from the stimulus of a wound has been shown to be essential if tumors are to form in crown gall.

The conditioning process, which renders the host cells susceptible to transformation, takes place independently of the bacteria at temperatures of both 25° C. and 32° C., although the cellular transformation is accomplished only at the lower temperature.

Conditioning, moreover, takes place gradually, reaching a maximum between the second and third days after a wound is made and declining again as wound healing progresses toward completion. If the host cells are not adequately conditioned, as appears to be the case in both the early and late stages of wound healing as well as in most normal cells not under the influence of wounded tissue, then the cellular transformation will not occur despite the presence in the tissues of many virulent crown-gall bacteria.

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Growth, 1952, **16**, 75-83.

THE INFLUENCE OF POTASSIUM CYANIDE ON GROWTH AND RESPIRATION IN *TETRAHYMENA GELEII*-FURGASON

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Introduction

Respiratory inhibitors are used in cellular studies in order to gain information concerning certain metabolic processes. They have been used in many investigations on unicellular organisms. For this purpose cyanide has been frequently employed. For some time ciliates were thought to be cyanide-resistant. However, Pace (10), Clark (3), and Boell (2), using balanced KOH-KCN solutions in the Barcroft-Warburg flasks as recommended by Krebs (7), demonstrated that some ciliates are cyanide-sensitive. Accelerating effects of cyanide upon aerobic respiration were observed in *Colpidium campylum* by Pitts (14) and in *Glaucoma piriformis* by Lwoff (9), but these could not be explained by the results of Pace, Clark and Boell. Recently, Pace and McCashland (13) found that the O_2 uptake of the giant amoeba, *Pelomyxa carolinensis*, may be increased by as much as 50% in a 10^{-11}M KCN solution and that growth might be increased by approximately 50% in 10^{-9}M KCN. Therefore, Pitts' and Lwoff's observations may be explained by the fact that, due to diffusion, a very low cyanide concentration which would cause acceleration of respiration is eventually reached. Dean (4) reports acceleration in the growth of *Tetrahymena* by low concentrations of cyanide, but no significant stimulating effect upon respiration in solutions of similar concentration. Dean utilized the direct method of Warburg in his respiratory studies. Therefore, conditions of his growth tests differed from those of his respiration studies, since in the latter, very little, if any, CO_2 was present in the Warburg flasks containing KOH-KCN mixture. Our investigations were designed to determine what effect original high or low cyanide concentrations would have upon growth and respiration in a ciliated form if the normally-present metabolite, CO_2, is not removed from the surrounding environment.

Materials and Methods

The organism used in these tests was *Tetrahymena geleii*-Furgason. Sterile cultures of this organism have been maintained in this laboratory in the same solution described for *Pelomyxa* (Pace and McCashland, (13)) containing 1% proteose peptone (Difco) and 1% glucose.

Stock cultures were maintained in cotton plugged, 16 x 150 mm., pyrex test tubes containing 5.0 ml. of the culture medium and were kept in a dark chamber at a temperature of 18 ± 2° C. Transfers were made every 3 to 7 days with a platinum wire loop.

Results

1. *Effect of Cyanide on Growth*

The tetrahymenas used in these tests were obtained from a 72-hour stock culture. A platinum loop which delivered 0.0223±0.0002 ml. of fluid was employed in transferring the organisms to the test media. Test tubes contained either 5.0 ml. of the medium for cultures to which no cyanide was added (controls), or 4.8 ml. of medium to which 0.2 ml. of sterile medium containing KCN was added to give the desired cyanide concentration. The concentrations varied from 10^{-2} to 10^{-12}M KCN. All tubes were stoppered with rubber vaccine vial ports (hollow center), a small opening having been made previously in the top with a heated needle and the hollow center filled with spun glass. This maintained constant pressure without allowing excessive loss of cyanide by diffusion, as occurred when cotton-plugged tubes were used. Culture tubes were incubated for 72 hours, in a dark chamber at 24° ± 1° C. The final culture population density was determined by use of a Klett-Summerson colorimeter (Elliott (6)), employing a blue filter. This method of enumeration was highly accurate in repeated readings involving comparisons of these counts with those of a series of culture dilutions. Results are presented in Table I. There is an increase in growth in 10^{-7}M KCN.

2. *Effect of Cyanide on Respiration*

From a flourishing 72-hour stock culture, 0.1 ml. was transferred to each 125 ml. pyrex Erlenmeyer flask containing 70 ml. of culture medium. The flasks were closed with cotton plugs, and were then incubated in the usual fashion for 72 hours at 24° ± 1° C. Cells from cultures of this type were used for respiration studies by the "indirect method of Warburg". This, rather than the "direct method", was

TABLE I

EFFECT OF KCN ON GROWTH IN *Tetrahymena Geleii*

*, Significant difference (P = <5%); **, highly significant difference (P = <2%); –, no significant difference (P = >5%)

Molar conc. KCN	No. of tests	Av. no. org./ml. (10^3)	Test as % of control
0	15	135	
10^{-2}	15	0	0
0	15	135	
10^{-3}	15	62	45.9
0	20	194	
10^{-4}	20	176	90.8**
0	15	135	
10^{-5}	15	114	84.4**
0	20	194	
10^{-6}	20	183	92.7**
0	10	143	
10^{-7}	10	154	107.6**
0	20	194	
10^{-8}	20	188	96.8*
0	35	169	
10^{-9}	37	164	97.0**
0	20	194	
10^{-10}	20	189	97.4–
0	10	134	
10^{-11}	8	135	100.7*
0	20	194	
10^{-12}	20	195	100.5–

chosen so that respiratory exchange of O_2 and CO_2 might be determined under conditions more nearly approximating those of the normal environment of the organisms (CO_2 present). For a single respiratory determination, a set of two flasks of approximately the same total volume was employed (8 sets were used for each experiment), but two different volumes of fluid were introduced. In one flask, 5.0 ml. of culture was used; in the other flask, 1.0 ml. This method was used, rather than dilution of 1.0 ml. of the culture to 5.0 ml., in order that the final cell concentrations would be the same. Thus errors due to altered ratios of cells to materials present (Dixon (5)) were avoided. For test determinations, cyanide was added to the cultures just before transferring the stated amount to

the flasks. All pipettes and flasks used were previously sterilized in an oven at 130° C. In the thermobarometers similar volumes of distilled water were employed. The temperature for all tests was held at 27° C. After a 15-minute equilibration period, the manometers were closed and readings were made at 30-minute intervals during a 2-hour period.

Since the rate of gaseous diffusion depends upon the surface of the fluid exposed, differences in surface area in flasks containing 5.0 and those containing 1.0 ml. of culture were corrected according to the method of Roughton (15). Solubility constants for oxygen and carbon dioxide were determined for the filtrate of a 72 hour culture. A sample of the filtrate and of distilled water was boiled to drive out dissolved gases. Equal volumes of either fluid were placed in Warburg flasks and the flasks attached to the manometers. The systems were flushed with oxygen for one minute, the stopcocks closed, and after 30 minutes the change in level of the manometer fluid was noted. By comparison with the solubility of oxygen in the distilled water ($\alpha = 0.02736$), solubility of oxygen in the filtrate was determined as $\alpha = 0.0209$. For determination of carbon dioxide solubility, boiled samples were used as before. The side arm of each flask contained 0.2 ml. of 2ON H_2SO_4. Carbon dioxide was passed through the system for two minutes and the systems closed. After 30 minutes the manometers were read and tipped so that the acid poured into the contents of the flask. After another 20 minutes a final reading was made. Again by comparison with carbon dioxide solubility in water ($\alpha = 0.718$) the value was calculated for the filtrate as $\alpha = 1.052$.

The results are presented in Table II. In 10^{-2}M KCN, complete inhibition of respiration was noted after 90 minutes. Concurrent with the Warburg tests, microscopical observations were made of the organisms from the control and test solutions. In 10^{-2}M KCN, motility was found to be retarded at 30 minutes; it was nearly stopped at 60 minutes, and only a few cells showed slight motility at 90 minutes. After 2 hours, there was no evidence of life in the organisms. The organisms continued to show life and movement in all the other concentrations tested. From these results it appears that respiration is not inhibited entirely by cyanide action upon the cytochrome-cytochrome oxidase (CCO) system. It is possible that the cells die of cumulative effects of cyanide on other systems and are not completely dependent upon CCO for their respiration. In 10^{-9}M KCN the rate

of O_2 consumption seems to be slightly accelerated as compared to the controls. No great difference was observed in the respiratory quotient of cells in the absence or presence of cyanide, except in concentrations of cyanide which were strongly inhibitory (10^{-2}M and 10^{-4}M KCN) in which cases the R.Q. value exceeds unity.

Discussion

Although these studies fail to show a great increase in rate of growth in Tetrahymena in low cyanide concentrations as reported by Dean (4), the slight acceleration of 10^{-9}M KCN upon respiration confirms the results reported by Pitts (14) and Lwoff (9) using *Colpidium campylum* and *Glaucoma piriformis,* respectively. Apparently these ciliated forms react in a manner similar to *Pelomyxa carolinensis* since a cyanide-resistant mechanism is present in each and seems capable of carrying out its metabolic processes at a more rapid rate under the influence of extremely minute concentrations of cyanide.

Conditions of the respiration studies were different from most of those previously reported for *Tetrahymena* and other closely related forms. However, the average O_2 uptake over a two-hour period for 120 control determinations was 181 µl./10^6 cells/hr. This approximates the values reported by Lwoff (8), Baker and Baumberger (1), and Pace and Lyman (12) when the differences in temperature, cell concentration and age of culture are considered. There was a slight decrease in the rate of O_2 uptake by cells as the tests progressed. Pace and Ireland (11) found that growth was inhibited in *Tetrahymena* in high concentrations of CO_2. It is likely that respiration is also affected in this way; that is, since there is an accumulation of CO_2 with the indirect method, the decrease in O_2 uptake may be due to this substance. On the other hand, Thomas (16) found that some CO_2 is actually utilized by *Tetrahymena* in the synthesis of succinate. Thus, metabolism should not be greatly inhibited by the presence of moderate amounts of CO_2.

The R.Q. values ascertained in these tests ranged from 0.94 to 0.82. Pace and Lyman (12) reported average respiratory quotients ranging from 0.99 to 1.07 for *Tetrahymena*. However, they did not use carbohydrates in their culture fluid. Also, they used the "direct method" in which one flask of a set contains KOH in the center well to absorb CO_2, while in the other flask the center well contains only water. Under these circumstances, abnormal environmental conditions are

produced in the flasks in which CO_2 is absorbed. The "indirect method", used in the present study, provides a more accurate determination under more nearly normal conditions. By means of the latter method the respiratory quotient is found to exceed unity only in cases where cyanide solutions exert a strongly inhibitory influence upon O_2 consumption. Even in these cases the values eventually return almost to normal.

In Figure 1, a graphic representation of O_2 consumption at different cyanide concentrations is shown. The results with the test cultures

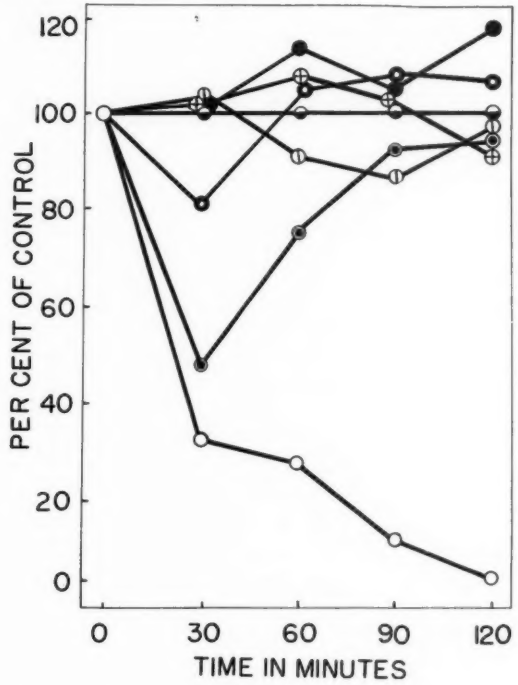

FIGURE 1

RELATIONSHIP BETWEEN OXYGEN CONSUMPTION IN *Tetrahymena Geleii* IN THE ABSENCE (CONTROL) AND PRESENCE OF CYANIDE.

◒, CONTROL; ○, 10^{-2}M; ⊙, 10^{-4}M; ◘, 10^{-6}M; ⓘ, 10^{-8}M; ●, 10^{-9}M; ⊕, 10^{-10}M.

are represented as a percentage of the control. These results indicate that while initial inhibition of respiration may be effected by high

cyanide concentrations, there is evidence of eventual partial recovery, except in 10^{-2}M KCN. However, although at this relatively high concentration there was a considerable decrease (75 per cent inhibition) in O_2 consumption during the first 30 minutes, it took 90 minutes longer for complete inhibition. At the same time, it may be noted that acceleration is not an immediate, but rather a slightly delayed occurrence. The curves for all the tests are somewhat similar where inhibition is concerned. This may be interpreted as further evidence for the possible existence of an adaptive mechanism (enzyme system) which becomes increasingly functional under the influence of cyanide. The fact that in 10^{-8}M KCN respiration falls below normal may possibly be explained as due to such a cyanide concentration that the cyanide-resistant fraction within the cell does not increase in function, whereas cyanide may still inhibit the cytochrome-cytochrome oxidase system. There is further evidence from the results presented in Figure 1 for 10^{-6} and 10^{-10}M KCN that a delicate balance exists within the cells. It may be that the cyanide-resistant fraction functions as a reserve, and if depleted, then cyanide again may be inhibitory. The respiratory quotient of cells in high cyanide concentrations also indicates a change in the nature of metabolism, since it rises above that of the controls during inhibition, but as recovery apparently occurs in the ability to utilize O_2, the respiratory quotient shows a tendency to return to a value nearer to that of the controls. The main reason for this change seems to lie in the rate of oxygen uptake, since carbon dioxide elimination seems to follow a more constant pattern (i.e., 10^{-4}M KCN, Table II).

The exact nature of the system by which the increase or recovery process occurs in the presence of cyanide is not known. The intent is to continue these investigations with the hope that some knowledge as to the mechanism involved may be obtained.

Conclusions

1. A method has been described for the study of growth in *Tetrahymena geleii*-Furgason in the presence of varying concentrations of KCN.

2. It was found that the cells of this species are killed in 10^{-2}M KCN while growth in 10^{-7}M KCN was approximately 8% greater than in cultures without cyanide.

3. The "indirect method of Warburg", with corrections for vari-

TABLE II

EFFECT OF KCN ON RESPIRATION IN *Tetrahymena Geleii*. O_2 CONSUMPTION AND CO_2 ELIMINATION REPRESENTED IN µl. PER MILLION CELLS FOR SUCCESSIVE 30 MINUTE PERIODS.

*, Significant difference (P = <5%); **, highly significant difference (P = <1%); –, no significant difference.

Molar conc. KCN	No. of tests	30′ O_2	30′ CO_2	30′ RQ	60′ O_2	60′ CO_2	60′ RQ	90′ O_2	90′ CO_2	90′ RQ	120′ O_2	120′ CO_2	120′ RQ
0	8	118	99	0.84	102	77	0.76	92	67	0.75	80	53	0.66
10^{-2}	8	39	43	1.10	29	34	1.17	8	7	0.88	0	0	—
0	23	81	75	0.93	84	72	0.86	84	77	0.92	68	55	0.81
10^{-4}	24	42	43	1.05	65	72	1.11	76	79	1.04	64	65	1.01
0	28	101	99	0.98	98	94	0.96	78	60	0.77	79	66	0.84
10^{-6}	28	83**	76	0.92	106**	101	0.94	85**	72	0.85	82–	74	0.90
0	15	86	76	0.88	90	80	0.88	92	76	0.83	85	78	0.92
10^{-8}	16	79–	73	0.79	87**	72	0.83	81*	69	0.85	80–	76	0.95
0	20	133	136	1.02	103	91	0.88	90	78	0.87	68	43	0.63
10^{-9}	20	131–	138	1.05	117**	108	0.92	95–	85	0.90	81**	62	0.77
0	24	116	107	0.92	102	95	0.93	80	74	0.93	89	82	0.92
10^{-10}	24	112	112	1.00	102	108	1.06	85	77	0.91	79	69	0.87
Av. of control	118	104.5	98.9	0.94	96.0	86.3	0.90	84.4	71.8	0.85	77.8	63.9	0.82

able conditions, has been utilized in ascertaining O_2 consumption and CO_2 elimination in *Tetrahymena*.

4. Low concentrations of KCN have been found to accelerate O_2 uptake, especially in 10^{-9}M concentration.

5. There is evidence of some degree of recovery from the immediate inhibitory effects of cyanide on respiration; this is noted chiefly after the first 30 minutes exposure.

6. R.Q. values range chiefly between 0.82 and 0.94 except in those organisms in which cyanide exerts a strong inhibitory action, where the values are greater than unity.

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EXPERIMENTAL ALTERATIONS OF CELL SIZE AND MITOTIC ACTIVITY IN THE OUTER ORBITAL GLAND OF THE WHITE RAT

V. *Observations on the Chemical and Biological Properties of the Mitosis Stimulating Agent in Homologous Tissue Extracts*

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In previous reports in this series the presence of a local mitosis-stimulating factor in the outer orbital gland of the rat was established. During tissue degeneration in vivo in the right outer orbital gland an increased mitotic activity was observed in the paired organ and in the homologous inner orbital glands (Teir, 1951a). During tissue degeneration, accompanied by active cell division, in heterologous tissues, a similar effect could not be demonstrated in the orbital glands (Teir, 1951b).

Intraperitoneal injections of aqueous extracts of mashed orbital gland tissue produced a highly mitosis-stimulating effect in all orbital glands, i.e. in homologous tissue (Teir, 1952). The mitoses were normal, a fact which excludes the possibility of any effect resembling that obtained in the orbital glands by means of colchicine, for instance (Teir, 1944, 1952).

The age of the donors and the receivers was of great importance. From younger animals a much more active extract was derived than from older ones. The sensitivity of the receiver to the extract also decreased with advancing age. The effective dose margin consisted mostly of some tenths of a milligram only. With an aqueous extract corresponding to 0.2-0.4 mg. of orbital gland tissue of 2-week-old rats a marked increase in mitotic activity was obtained, the optimum occurring 2 days after application in rats about 2 months old. A few milligrams more or less of tissue extract usually did not give positive results.

The purpose of the present experiments was to obtain a preliminary idea of the nature of the active agent. The method was the same as

before. The orbital gland was removed immediately after the animal had been decapitated, and weighed and mashed in a mortar together with 0.5 ml. of distilled water. After the mashing a further quantity of distilled water was added so that 1 ml. of extract suspension corresponded to 1 mg. of orbital gland tissue. The basic extract thus obtained was subjected to various kinds of treatment but was always made up with distilled water to the original volume. From this extract further dilutions were made in order to obtain the concentrations which had proved effective in previous experiments (Teir, 1944).

The extracts were injected at the same time of day and the animals lived under exactly the same conditions during the time of the experiment. They were decapitated at the same time of day, provided a shorter time of influence was not required. The orbital glands were immediately fixed in Bouin's fluid. The 5 μ thick sections were stained in Heidenhain's iron hematoxylin. The mitotic rate was determined by counting the number of mitoses in one hundred fields, chosen at random, with 40× objective and 10× ocular.

Under normal conditions solitary mitoses are found in the outer orbital glands of 6-8-week-old rats (Fig. 1), so that a mitotic rate above 10 was considered higher than normal. In old rats, 7 to 9 months old, isolated mitoses are normally found to be extremely rare (Teir, 1944, 1952).

Water-solubility

The question of the water-solubility of the active agent is of the greatest importance apropos of transport in the organism.

The extract was made from five 10-day-old rats of the same litter weighing 17 to 19 gr. A total of 39.5 mg. of orbital gland tissue was obtained. Of the basic extract 20 ml. were centrifuged (2000 r.p.m.) for 20 minutes whereby a clear cell-free fluid and a sediment were obtained which were separated with a pipet. To the sediment 20 ml. of distilled water were added. The solutions injected were (a) untreated basic extract, (b) the clear fluid obtained by centrifugation, (c) the sediment obtained by centrifugation, and (d) the clear fluid reunited with the sediment obtained by centrifugation.

The result of the investigation is given in Table 1. Group A showed a clear, although not very marked, mitotic increase in one animal which had received 0.25 mg. while the other two animals showed normal values.

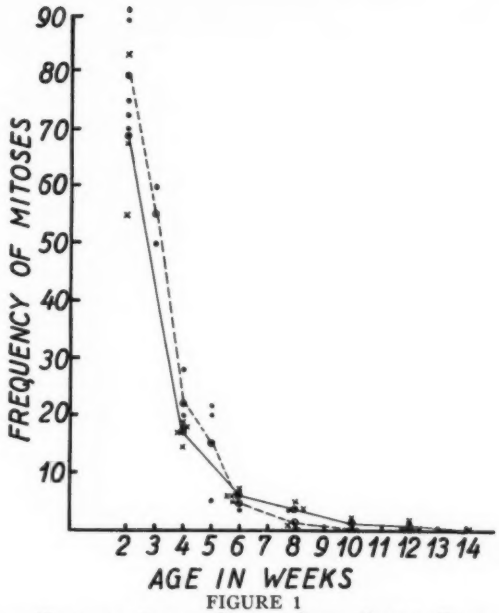

FIGURE 1

MITOTIC RATE AT DIFFERENT AGES IN NORMAL OUTER ORBITAL GLAND OF THE WHITE RAT AT TWO DIFFERENT STOCKS

The interrupted line indicates the stock used in these experiments.

In group B a very marked mitotic peak was obtained with 0.5 mg. and there was also a clear increase in one of the animals that had been given 0.25 mg. while the other did not react to the extract.

Group C shows an increased mitotic rate in all animals.

In group D a positive result was obtained only in one of the animals that received 0.25 mg.

The result in group B indicates that the agent is water-soluble or that it occurs in cell-free water suspensions. Comparing the four groups, we find that the smallest mitotic increase was obtained with the basic extract and with the similar extract in group D. The positive result in group C may be explained by the fact that the extract contained pieces of tissue which might not have emitted all the active agent into the solution. The considerably poorer result in group D might possibly depend on the presence of an inhibiting factor with different solubility properties from the stimulating factor.

TABLE 1

ACTIVITY OF THE MITOSIS-STIMULATING ORBITAL GLAND AGENT IN AN AQUEOUS ENVIRONMENT

Groups of experiments	Rats			Extracts			Mitotic ratio			
							Outer orbital gland		Inner orbital gland	
	No.	Age Months	Body-weight	Amount mg.	Time of influence Hours	Different extraction fractions	Right	Left	Right	Left
A	685	2½	165	0.50	48	Control of extracts in the usual way	3	2	2	0
	686	"	152	0.25	"		3	2	1	5
	687	"	103	"	"		18	12	20	25
B	688	"	125	0.50	"	Water-soluble fraction	130	148	99	98
	689	"	190	0.25	"		4	2	2	5
	690	"	91	"	"		24	27	10	5
C	691	"	104	0.50	"	Non-water-soluble fraction	15	30	4	12
	692	"	80	0.25	"		44	31	15	45
	693	"	132	"	"		15	6	5	3
D	717	"	72	0.50	"	Water-soluble and non-water-soluble fractions reunited	3	1	14	3
	718	"	74	0.25	"		0	1	2	2
	719	"	75	"	"		22	10	8	10

Solubility in Chloroform

The solubility of the active agent in chloroform was investigated by two different methods. In the first it was extracted from an aqueous solution and in the second it was extracted directly with chloroform from orbital tissue.

To 18 ml. of a basic extract, which proved very active, 10 ml. of neutral chloroform were added and the solutions were then shaken for 10 minutes. In the chloroform portion there was a dense sediment. The water remained somewhat turbid and opalescent. Between these two layers was a layer, some millimeters in thickness, consisting of a deposit resembling ice crystals. This layer and the chloroform portion were separated off. To the water 6 ml. of chloroform were added and the solution was shaken for a further 10 minutes. A fairly heavy sediment was formed and the water became clear. The chloroform was added to the water and now both the chloroform and the water remained clear. The chloroform was separated off. The remaining water was used as a basic extract in group A in this series (Table 2).

The middle layer with ice-like crystals was evaporated and a membranous formation was obtained. This was finely ground and 19 ml. of distilled water were added. The suspension thus obtained was given to group B.

The different chloroform fractions were evaporated together, and washed in 20 ml. of distilled water. Chloroform was again added, and evaporated. Twenty ml. of 95 per cent alcohol were first added and then 20 ml. of distilled water. The alcohol was separated by vacuum distillation, after which 1 ml. of fluid remained. To this 19 ml. of distilled water were added. An opalescent fluid was obtained and this chloroform-soluble fraction was used in group C.

The result of the experiment is given in Table 2. From an aqueous extract of orbital gland tissue the active agent can obviously not be totally extracted with chloroform since a markedly positive result was obtained with 0.5 mg. (No. 694). On the other hand the positive result in No. 701 indicates that the agent is also soluble in chloroform and a direct extraction of orbital gland tissue with chloroform therefore proved necessary. The middle layer from the first contact between aqueous extract and chloroform did not give a positive result.

For direct extraction of orbital gland tissue with chloroform the

TABLE 2

SOLUBILITY IN CHLOROFORM OF THE MITOSIS-STIMULATING ORBITAL GLAND AGENT

Groups of experiments	Rats			Extracts			Mitotic ratio			
							Outer orbital gland		Inner orbital gland	
	No.	Age Months	Body-weight	Amount mg.	Time of influence Hours	Different extraction fractions	Right	Left	Right	Left
A	694	2½	120	0.5	48	Chloroform and water extract. Water-soluble portion.	92	38	57	50
	695	"	90	0.25	"		21	12	11	20
	696	"	119	"	"		9	11	4	2
B	697	"	120	1.0	"	Middle layer.	1	7	—	2
	698	"	145	0.5	"		0	7	5	3
	699	"	100	0.25	"		3	6	6	9
C	700	"	142	1.0	"	Chloroform-soluble portion in water-suspension.	4	6	1	1
	701	"	110	0.5	"		25	51	5	4
	702	"	98	0.25	"		3	6	6	8
	714	1½	68	1.0	"		0	6	—	7
	715	"	73	0.5	"		2	1	14	18
	716	"	71	0.25	"		0	7	8	5
D	703	2½	129	1.0	"	Direct extraction with chloroform. Chloroform-soluble portion.	4	5	7	7
	704	"	98	0.5	"		5	6	5	1
	705	"	103	0.25	"		35	23	33	9
	706	1½	77	1.0	"		3	9	30	16
	707	"	76	0.5	"		9	7	12	2
	708	"	69	0.25	"		6	7	25	23
E	709	2½	78	0.5	"	Portion insoluble in chloroform.	2	2	3	9
	710	"	110	0.25	"		10	11	13	—
	711	1½	69	0.5	"		2	0	23	2
	712	"	85	0.25	"		8	8	9	7
	713	"	75	1.0	"		6	8	6	14

outer orbital glands of two-week-old rats weighing 18-19 gr. were used. The 22 mg. of orbital gland tissue thus obtained was mashed in a mortar with 1 ml. of chloroform, after which 19 ml. of chloroform were added. The extract was centrifuged (2000 r.p.m.). The chloroform was evaporated and 20 ml. of 95 per cent alcohol and 20 ml. of distilled water were added. The alcohol was separated by vacuum distillation, leaving 2 ml. of fluid, to which 20 ml. of distilled water were added. The fraction thus obtained was used for a basic extract in group D.

The sediment obtained on centrifugation of the chloroform extract was evaporated and 20 ml. of 95 per cent alcohol and 20 ml. of distilled water were added. The alcohol was separated by vacuum distillation, and 2 ml. of fluid remained to which 20 ml. of distilled water were added. The fraction thus obtained, which was insoluble in chloroform, was used in group E.

It appears from Table 2 that three animals in group D showed a positive result, which indicates that the agent is soluble in chloroform. In group E we could also observe some clear, although faint, positive results. These may be explained on the assumption that not all the active agent was extracted before the centrifugation but that part of it was extracted during the later treatment of the sediment.

Solubility in Ether

From three 11-day-old rats weighing 20, 19 and 20 gr. the outer orbital glands were removed after decapitation. Together they weighed 35.5 mg. They were mashed in a mortar together with ether and the tissue dissolved better than in any of the previous experiments. Thirty-five ml. of ether were added and the mixture was centrifuged for 15 minutes (2000 r.p.m.). The ether portion and 35 ml. of distilled water were vacuum distilled, after which 35 ml. of opalescent fluid remained. The sediment was washed with ether and evaporated and 35 ml. of distilled water were added.

The experiments indicated that the active agent is insoluble in ether (Table 3).

Influence of Heat

In these experiments the age was varied and in consequence the following main groups were set up: (1) extract of young animals to growing rats and (2) to old rats and (3) extract of old rats to young ones.

TABLE 3

SOLUBILITY IN ETHER OF THE MITOSIS-STIMULATING ORBITAL GLAND AGENT

Rats			Extracts			Mitotic ratio			
						Outer orbital gland		Inner orbital gland	
No.	Age Months	Body-weight	Amount mg.	Time of influence Hours	Different extraction fractions	Right	Left	Right	Left
736	2	104	1.0	48	Direct extraction with	0	0	2	4
737	"	121	"	"	ether. Soluble portion.	1	0	1	7
738	"	130	0.5	"		3	2	2	3
739	"	134	"	"		0	0	—	1
740	"	98	0.25	"		2	0	0	0
741	"	100	"	"		1	2	8	2
742	"	81	0.1	"		0	0	4	0
743	"	110	"	"		1	1	8	10
744	"	105	1.0	"	Portion insoluble in	0	0	0	0
745	"	110	"	"	ether.	1	3	0	0
746	"	83	0.5	"		5	8	5	6
747	"	75	"	"		5	2	1	2
748	"	82	0.25	"		2	6	—	6
749	"	95	"	"		18	13	13	14
750	"	95	0.1	"		16	9	11	7
751	"	80	"	"		7	14	5	18

Twenty degrees C. corresponded to room temperature and was mainly used as a control for experiments with higher temperatures. By means of water-baths a temperature of 60° C. was obtained, while 100° C. was reached by keeping the extract in thin glass tubes in boiling water. In order to observe the influence of 200° C., 20 ml. of the basic extract were kept under a pressure of 16 atmospheres in a glass ampule which would hold 80 ml. in a gas oven. After 15 minutes at 200° C. the originally highly opalescent fluid became only slightly opalescent. After 1 hour and 3 hours at this temperature the fluid was quite clear.

Extract of Young Animals to Growing Rats

In the control experiments (Table 4, group A and C) a marked stimulation of mitotic cell division was obtained with 0.4 mg. of extract.

After treatment for 15 minutes at 100° C. (group B) the extract was still active. The mitotic rate was even greater than usual, doses as large as 1.6 mg. and 0.8 mg. giving equally good results as the generally most active doses of 0.4 and 0.2 mg. Neither was the difference between animals killed 24 and 48 hours after treatment as large as usual; there were already numbers of mitoses after 24 hours.

After 15 minutes, from a biological point of view, in the very high temperature of 200° C., the effectiveness of all concentrations persisted (Table 4, group D). But the reversal in the relation between the mitotic rate and the quantity of mashed tissue after this heat treatment was remarkable. With untreated extract the best effect was obtained with 0.4 mg.; with 0.2 and 0.1 mg. it decreased. The effect was greatest after heating with 0.1 mg., and with 0.4 mg. it was lower than in the animal which showed a positive result with 0.2 mg. Since, however, only a small number of animals was involved, individual variations might even be of decisive importance.

After 60 minutes at 200° C. the extract showed a mitosis-stimulating effect in one animal only.

After 3 hours at 200° C. the result (Table 4, group F) was surprising since a clearly positive result was obtained with 0.2 and 0.1 mg. of tissue (No. 734, 735).

Extract of Young Animals to Old Rats

These experiments can be divided into three groups (Table 5, group A, B and C).

TABLE 4

INFLUENCE OF TEMPERATURE ON EXTRACTS OF YOUNG ANIMALS GIVEN TO GROWING RATS

Groups of experiments	Rats			Extracts			Time of influence	Mitotic ratio			
					Temperature			Outer orbital gland		Inner orbital gland	
	No.	Age Months	Body-weight	Amount mg.	°C.	Time, min.	Hours	Right	Left	Right	Left
A	593	2	75	1.7	20	15	24	6	3	1	0
	594	"	102	"	"	"	48	1	2	0	0
	595	"	64	0.8	"	"	24	0	1	1	2
	596	"	96	"	"	"	48	27	13	12	7
	597	"	115	0.4	"	"	24	10	1	12	12
	598	"	75	"	"	"	48	141	132	74	69
	599	"	80	0.2	"	"	24	6	24	11	7
	600	"	64	"	"	"	48	22	14	6	6
B	601	"	62	1.7	100	"	24	43	36	14	0
	602	"	95	"	"	"	48	14	8	3	4
	603	"	74	0.8	"	"	24	12	16	11	2
	604	"	66	"	"	"	48	24	31	12	23
	605	"	71	0.4	"	"	24	25	15	14	17
	606	"	100	"	"	"	48	65	42	10	3
	607	"	92	0.2	"	"	24	13	17	40	39
	608	"	86	"	"	"	48	23	30	12	6
C	720	"	67	0.4	20	"	40	61	61	125	51
	721	"	70	0.2	"	"	"	24	18	21	17
	722	"	48	"	"	"	"	18	13	8	4
	723	"	47	0.1	"	"	"	4	6	9	8

TABLE 4 (*continued*)

Groups of experiments	Rats			Extracts			Mitotic ratio			
							Outer orbital gland		Inner orbital gland	
	No.	Age Months	Body-weight	Amount mg.	Temperature °C.	Time, min.	Time of influence Hours	Right	Left	Right	Left
D	724	"	52	0.4	200	"	"	14	24	3	4
	725	"	44	0.2	"	"	"	2	1	3	1
	726	"	52	"	"	"	"	26	42	11	8
	727	"	49	0.1	"	"	"	75	86	12	91
E	728	"	61	0.4	"	60	"	8	4	36	9
	729	"	51	0.2	"	"	"	0	0	1	3
	730	"	72	"	"	"	"	1	4	4	2
	731	"	34	0.1	"	"	"	0	0	0	0
F	732	"	61	0.4	"	180	"	0	0	1	1
	733	"	51	0.2	"	"	"	0	0	3	1
	734	"	58	"	"	"	"	27	22	9	23
	735	"	41	0.1	"	"	"	6	37	10	26

In group A a large quantity of orbital gland tissue from 12-day-old rats was given to 7-month-old rats. The controls showed a slight but clear mitotic increase as early as 4 hours afterwards (No. 525). Of the greatest interest, however, are the high mitotic values after 2 days with extracts exposed to 60° C. and 100° C. for 15 minutes (No. 530 and 533). I have never found in the outer orbital gland of adult rats a mitotic rate as high as in the latter.

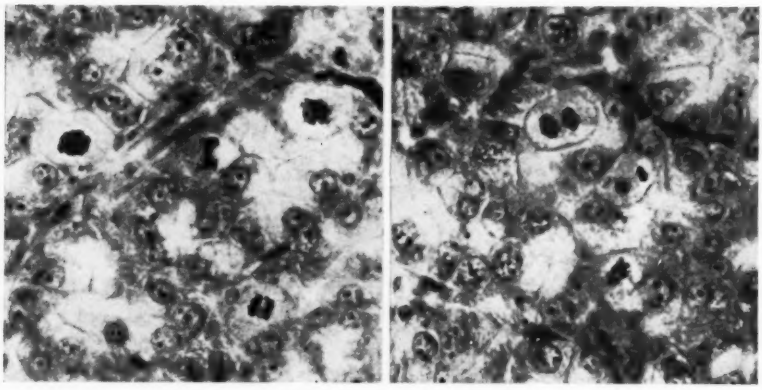

FIGURE 2
HIGH-GRADE MITOTIC ACTIVITY IN THE OUTER ORBITAL GLAND TWO DAYS AFTER INTRAPERITONEAL INJECTION OF HOMOLOGOUS EXTRACT EXPOSED TO 100° C. FOR 15 MINUTES INTO ONE 7-MONTH-OLD RAT (× 340).

In group B the extract was also prepared from 12-day-old rats. The tissue was mashed in a mortar without addition of water during the mashing. The basic extract was kept for 15 minutes at 100° C. The quantity varied, whereas the time of influence. 48 hours, was the same for all animals. The rats were 9 months old. There was a slight positive effect in some animals but no peaks in the mitotic rate occurred (Table 5, group B).

In group C the extract was again prepared from 12-day-old rats, and injected into 9-month-old rats (Table 5, group C). The same extract had been injected into 2-month-old rats and proved very active (Table 4, Nos. 593-608). In this experiment, however, a slight mitotic increase was observed in one animal only (No. 580) 24 hours after application.

TABLE 5

INFLUENCE OF TEMPERATURE ON EXTRACTS OF YOUNG ANIMALS GIVEN TO OLD RATS

Groups of experiments	Rats			Extracts			Mitotic ratio			
					Temperature			Outer orbital gland		Inner orbital gland	
	No.	Age Months	Body-weight	Amount mg.	°C.	Time, min.	Hours	Right	Left	Right	Left
A	525	7	170	12.0	20	15	4	10	6	11	—
	526	"	214	"	"	"	19	0	0	0	0
	527	"	270	"	"	"	48	4	2	2	2
	528	"	155	"	60	"	4	0	0	0	0
	529	"	118	"	"	"	19	1	0	0	1
	530	"	180	"	"	"	48	65	—	13	—
	531	"	120	"	100	"	4	12	8	3	7
	532	"	280	"	"	"	19	3	1	3	1
	533	"	145	"	"	"	48	112	108	32	12
B	556	9	280	7.4	100	"	48	0	0	0	0
	567	"	245	"	"	"	"	1	3	0	3
	568	"	280	"	"	"	"	2	0	3	0
	569	"	260	"	"	"	"	1	5	1	0
	570	"	190	1.8	"	"	"	—	—	0	0
	571	"	295	"	"	"	"	1	0	0	0
	572	"	305	0.4	"	"	"	1	1	0	0
	573	"	260	"	"	"	"	2	0	0	0
	574	"	245	0.1	"	"	"	—	4	8	2
	575	"	260	"	"	"	"	2	0	1	0
	576	"	195	0.003	"	"	"	4	—	1	15
	577	"	288	"	"	"	"	0	1	0	0

TABLE 5 (*continued*)

Groups of experiments	Rats			Extracts			Time of influence Hours	Mitotic ratio			
					Temperature			Outer orbital gland		Inner orbital gland	
	No.	Age Months	Body-weight	Amount mg.	°C.	Time, min.		Right	Left	Right	Left
C	578	"	246	3.5	20	"	[illegible]	0	0	1	0
	579	"	330	"	"	"	[illegible]	2	1	1	1
	580	"	260	"	"	"	24	8	5	2	3
	581	"	262	"	"	"	"	2	3	0	0
	582	"	290	"	"	"	48	[illegible]	1	0	0
	583	"	232	"	"	"	96	[illegible]	0	0	0
	584	"	228	"	100	"	10	0	1	1	0
	585	"	265	"	"	"	"	0	0	1	0
	586	"	190	"	"	"	24	0	0	0	0
	587	"	165	"	"	"	"	0	1	0	0
	588	"	280	"	"	"	48	1	2	0	0
	589	"	236	"	"	"	"	2	0	0	0
	590	"	294	"	"	"	"	2	1	0	0
	591	"	160	"	"	"	96	0	0	0	0
	592	"	250	"	"	"	"	0	0	0	0

TABLE 6

INFLUENCE OF TEMPERATURE ON EXTRACTS OF OLD RATS GIVEN TO OLD RATS

Rats			Extracts			Time of influence	Mitotic ratio			
				Temperature			Outer orbital gland		Inner orbital gland	
No.	Age Months	Body-weight	Amount mg.	°C.	Time, min.	Hours	Right	Left	Right	Left
548	10	250	22	20	15	1	0	1	0	0
549	"	200	"	"	"	2	0	0	0	0
550	"	215	"	"	"	4	0	1	1	0
551	"	190	"	"	"	15	0	0	0	0
552	"	220	"	"	"	26	0	0	0	0
553	"	230	"	"	"	48	0	0	0	0
554	"	188	"	"	"	96	0	2	13	0
555	"	280	"	"	"	168	0	0	0	0
565	"	120	11	"	"	48	12	23	14	20
556	"	175	22	100	"	2	0	0	0	0
557	"	200	"	"	"	15	0	0	0	0
558	"	175	"	"	"	26	0	0	0	0
559	"	160	"	"	"	48	1	0	0	0
560	"	175	"	"	"	96	0	0	0	0
561	"	220	"	"	"	168	0	0	0	0
562	"	204	"	"	"	216	0	0	0	0
563	"	160	"	"	"	264	1	0	1	1
564	"	155	11	"	"	48	29	46	4	35

Extract of Old Rats to Old Animals

Extract of four 10-month-old rats was injected into rats of the same age (Table 6). The dose was 22 mg. Two rats which received 11 mg. were the only animals that showed a clear mitotic increase. The rat receiving extract exposed for 15 minutes to 100° C. showed a higher mitotic rate than the rat receiving untreated extract.

These experiments show that the active agent in the orbital gland extract is very thermostable and often proved to be even more active after heat treatment.

Preservation in Aqueous Solution

An extract of orbital gland tissue from a 2-week-old rat which had proved very active (Teir, 1952; Table 6, Nos. 609-626), was hermetically sealed and kept at +4° C. for 3 weeks and was thereafter injected into 2-month-old rats (Table 7). With 0.4 mg. a slight mitotic increase was obtained in two animals, while 0.2 mg. did not give any clear positive result. The experiment indicates that the effect decreases when the water extract is stored.

TABLE 7
Effect of Extract Preserved in Aqueous Solution 3 Weeks at +4° C.

Rats			Extracts		Mitotic ratio			
					Outer orbital gland		Inner orbital gland	
No.	Age Months	Body-weight	Amount mg.	Time of influence Hours	Right	Left	Right	Left
679	2	137	0.2	48	0	2	5	4
680	"	99	"	"	0	0	4	2
681	"	120	"	"	3	1	0	3
682	"	80	0.4	"	1	9	1	9
683	"	106	"	"	—	11	3	3
684	"	69	"	"	0	1	0	2

Effect of Dried Orbital Gland Tissue

The right orbital glands of two 2-week-old rats weighed 3.2 mg. and 2.4 mg. They were placed between two watch-glasses and were kept at room temperature for 24 days. The drying was not made more effective since Fischer (1941) and Fischer and Astrup (1943), when drying the agent in an extract of embryonic tissue active as a growth-promoting factor in tissue cultures, found it important that the drying should not be too thorough.

When dry, the glands weighed 1.0 and 0.5 mg. They were crushed in a mortar together with a few drops of water, and distilled water was

then added until the usual basic extract was obtained. The extract was kept for 24 hours at room temperature before it was injected into 2-month-old rats, all of which were killed two days later.

With orbital gland tissue dealt with this way, a clear, though slight, stimulation of mitotic cell division was obtained (Table 8).

TABLE 8
EFFECT OF EXTRACT OF DRIED ORBITAL GLAND TISSUE

Rats			Extracts		Mitotic ratio			
					Outer orbital gland		Inner orbital gland	
No.	Age Months	Body-weight	Amount mg.	Time of influence Hours	Right	Left	Right	Left
669	2	132	1.5	48	14	7	7	8
670	"	138	"	"	4	4	22	13
671	"	72	0.75	"	0	4	2	0
672	"	135	"	"	2	0	2	3
673	"	114	0.4	"	11	12	2	6
674	"	92	"	"	3	8	2	2
675	"	91	0.2	"	3	2	2	4
676	"	114	"	"	4	6	15	8
677	"	116	0.1	"	1	0	—	2
678	"	154	"	"	3	1	7	7

DISCUSSION

The experiments performed in this series support the belief that in the outer orbital gland of the rat there is a mitosis-stimulating agent which is activated by mashing orbital gland tissue in vitro.

From rats about 12-14 days old orbital gland extracts were obtained which were active in doses of 0.5 to 0.2 mg. with an optimal effect about two days after intraperitoneal injection into 5-8-week-old rats (Table 1-4, 8). With a 20 to 30 times larger dose a marked mitotic stimulation was also obtained in old animals (Table 5). With about the same dose of 10-month-old orbital gland tissue a similar effect was also obtained in 10-month-old rats (Table 6).

The different extracts often showed a surprisingly high mitosis-stimulating effect after various treatments in vitro, which indicates that the agent is very stable.

It is interesting to compare the properties of this active agent with other cell division and cell multiplication stimulating agents occurring in the vegetable and animal kingdom, viz. auxin, hetero-auxin, botanical wound hormones, the cancer virus agent, the induction agent, growth-promoting substances in embryonic extracts active in tissue cultures and the osteogenic factor (Levander, 1938; Annersten. 1940) which occurs in extracts of bone tissue and which, when injected

intramuscularly, induces pluripotent connective tissue cells to metaplastic bone growth.

The experiments show that the active agent in the orbital gland extracts is soluble in water, or occurs in cell-free water suspensions. The same applies to the above-mentioned botanical agents (see Went, 1937; Thimann and Went, 1949). The cancer virus agent is active in ultravisible water extracts, as was proved by Rous as early as 1911. The induction agent, too, is active in aqueous environment (Spemann, Fischer and Wehmeier, 1933; Holtfreter, 1934; Needham, Waddington and Needham, 1934) and so is the active factor in the embryonic extracts which stimulate growth in tissue cultures (see Fischer, 1946). The osteogenic factor is probably water-soluble also (Annersten, 1940). A feature common to all these different cell division stimulating agents is thus their activity in cell-free water solutions and suspensions.

In its relation to fat solvents the orbital gland agent differs from these other agents in several respects. The fact that it does not apparently dissolve in ether distinguishes it from auxin, which dissolves in ether, although fairly slowly (Gustafson, 1941; Thimann and Skoog, 1940). Some investigators (Needham, Waddington and Needham, 1934) have found the induction agent to be ether-soluble, while others have observed that various tissues still have a strong induction effect after ether treatment (Spemann, Fischer and Wehmeier, 1933; Wehmeier, 1934; Holtfreter, 1934). The osteogenic factor is probably ether-soluble (Annersten). The cancer virus agent is also mostly considered ether-soluble. There seems to be some uncertainty as to the ether-solubility of these different agents. Only the active agent in embryonic extracts active in tissue cultures is actually not ether-soluble (Fischer, 1951, personal information).

Like the orbital gland tissue agent, most of these other agents are considered soluble in chloroform.

Another property that seems to be common to the different agents is their instability in aqueous solutions. With regard to auxin this depends on the extreme sensibility to oxidative destruction (Thimann, 1934). The embryonic extracts active in tissue cultures lose part of their activity in the course of 1-2 weeks even at constant pH and when kept in the refrigerator (Fischer, 1946).

One property common to all these agents seems to be their resistance to drying. However, in material in which the auxin can at first

be liberated it can be made unextractable by drying (Thimann and Skoog, 1942). The active agent in Rous sarcoma is active after drying (Rous and Murphy, 1914; Hoffstadt and Tripi, 1946; Carr and Harris, 1951, and others). The growth-promoting substances active in tissue cultures do not lose their effect even after prolonged storage in a dry condition (Borger and Zenker, 1932; Peacock and Shukow, 1940; Hetherington and Craig, 1940; Fischer, 1941; Fischer and Astrup, 1943). The induction agent is also active after drying (Holtfreter, 1932, 1934; Bautzmann, 1932). Annersten's negative results with regard to the reaction of the osteogenic factor to drying may not perhaps be quite conclusive, owing to too limited material.

The orbital gland agent differs from several other cell division stimulating agents in its high thermostability. The induction agent endures boiling for some time without loss of activity (Bautzmann, 1932; Holtfreter, 1932; Waddington, 1933). In a dry condition the induction agent withstood heat treatment of up to 135° C., but heating to 150° C. for one hour destroyed the effect (Holtfreter, 1934). Annersten found that the osteogenic factor could withstand boiling and this was also proved by Went (1926, 1928) with regard to auxin. Other agents have mostly been considered rather thermolabile. Carrel (1912-13), for instance, found that the embryonic extract active in tissue cultures lost two-thirds of its effect after 10 minutes' heating to 56°. According to Fischer (1940), however, a thermostable substance can also be extracted from the hearts of calf embryos. At 56° C. the cancer virus agent loses its activity. The Shope papilloma agent, however, endures heating to 65° C. (Shope, 1933).

The experiments in which the orbital gland extracts were boiled gave even better results than those in which untreated extracts had been used, and at the same time a widening of the narrow effective dose margin and of the time of influence could be observed (Table 4). In one of these experiments mitotic values were obtained that had never before been produced in old animals with untreated extracts. In this respect, also, the agent resembles the induction agent, viz. through boiling, non-inductors can be made active (Holtfreter, 1933, 1934). Thus Holtfreter found that the growth-promoting factors in the embryonic extracts active in tissue cultures do not induce until denatured by boiling. By means of heat treatment Toivonen (1950) also proved a regional change in the induction capacity of his specific heterologous inductors.

It is possible that the mashing of the orbital gland tissue activates an agent which occurs in normal intact tissue in a more or less inactive form like an auxin for instance (see Kögl, Erxleben and Haagen-Smit, 1934) and botanical wound hormones (English, Bonner and Haagen-Smit, 1939). This activation seems to be further accentuated by heat treatment. It is possible that the active agent, in the same way as the botanical agents, is accompanied by co-factors inactive in themselves which enhance the effect. In the same way as with the orbital gland extracts, English, Bonner and Haagen-Smit used mashed tissue and were able to isolate a substance in a crystalline form which they called traumatic acid, whose effect was enhanced to a marked degree by the presence of substances in themselves inactive, such as glutamic acid and sucrose.

Both in the heat experiments and in the experiments on water-solubility, observations were made which indicated that an inhibition might be attached to the active agent which, among other things, was not as thermostable as the stimulator. Marshak and Walker (1945) proved that such an inhibitor was active along with a stimulator of cell division in the regenerating liver of hepatectomized rats after intraperitoneal injections of homologous chromatin fractions. These two factors were distinguished from one another by different solubility properties. In the liver also the activity of the inhibitor can clearly be eliminated by heat treatment (Brues, Subbarow, Jackson and Aub, 1940). In connection with other discussions on cell multiplication, too, a simultaneous occurrence of a thermostable and a thermolable factor is mentioned. According to Menkin the rise in the number of circulating leukocytes with inflammation is referable to at least two factors released by the cells injured at the site of the injury, a thermolabile leukocytosis-promoting factor and a thermostable leukocytosis factor, of which the latter has now been isolated in a crystalline form (Menkin, 1950).

The presence of mitosis-inhibiting factors in the orbital gland, liver and blood-forming organs seems to have a parallel also during normal embryonic development. Spemann, Fischer and Wehmeier (1933) proved the presence of an inhibitor, "Hemmungsstoff", active in induction. Like the assumed inhibitor in the orbital gland, this too, is thermolabile.

A summary of this comparison between the orbital gland agent and the other plant and animal mitosis-stimulating agents shows that the

former has many properties in common with several of the agents mentioned here. However, it resembles most nearly the induction agent which, from the point of view of development, is quite natural. It also has several features in common with the growth-promoting substances in extracts of embryonic animal tissue active in tissue cultures.

The two latter agents are, however, neither tissue- nor species-specific (see Needham, 1950; Fischer, 1946). Investigations on tissue specificity which are at present being made in our laboratory and which will be dealt with in a later report in this series, indicate a far advanced tissue specificity. In this respect the effect resembles that of known hormones.

A similar mitosis-stimulating tissue factor was also demonstrated in the skin of the rat (Teir, Kiljunen and Putkonen, 1951) which, when injected intraperitoneally, influences the time of wound-healing and the new formation of epithelium in experimental wound-healing investigations (Teir, Putkonen and Kiljunen, 1951) and it can therefore be compared with the wound hormones discussed in similar connections. Judging from preliminary investigations (Teir and Kiljunen, 1952) this skin factor seems also to be species-specific and thus, in this respect, it differs from the usual concept of a hormone. The latter investigation, however, gave results which argue in favor of the possibility that extracts of human skin cancer tissue may influence the mitotic ratio in the normal skin of the rat. It is possible that the corresponding agents in malignant tissue are less specific and "chemically pluripotent", thus possessing properties lacking in normal postembryonic tissue but possibly occurring in normal embryonic tissue. This hypothesis resembles Huxley's (1924) assumption that morphological differentiation during normal development constitutes a visible result of an invisible chemical differentiation.

The orbital gland tissue agent actually has this tissue and species specificity in common only with the active agent of cancer virus. Here the species specificity is not absolute either, Rous sarcoma, for instance, can be transferred to other fowl than chickens (Duran-Reynals, 1943, 1947) and can be made to grow even in the anterior chamber of the eye of the guinea pig (Shrigley, Greene and Duran-Reynals, 1945, 1947). Like the orbital gland agent, the active cancer virus agent seems also to be accompanied by an inhibitor (see Claude, 1937). Apart from its thermostability and ether-solubility, the orbi-

tal gland agent thus seems to have many properties in common with the active agent in cancer virus.

The present experiments do not allow us to draw definite conclusions as to the chemical nature of the orbital gland agent or its point of attack in the cell. However many factors argue that the active principle may be looked for, as is the case with induction agents (see Kuusi, 1951), in the group of nucleoproteins, and investigations are being continued on this line.

The positive mitotic stimulation obtained with homologous tissue extracts even when treated in various ways in vitro, seems to support my growth theory (Teir, 1951a). According to this theory postembryonic cell multiplication and cell growth is controlled, at least in higher vertebrates, by a local peripheral system on the one hand, to which this mitosis-stimulating tissue factor could be referred, and by a superior system on the other hand, in which the growth hormone of the anterior hypophyseal lobe and other known endocrine organs may be of importance.

Summary

1. Through intraperitoneal injections of extract of mashed orbital gland tissue a remarkable stimulation of the capacity for mitotic cell division in homologous organs was obtained.

2. The agent in the extracts was found to be active in an aqueous environment, to be soluble in chloroform, apparently insoluble in ether and very thermostable. Its effectiveness persists in dried tissue but is lost in aqueous solutions on keeping.

3. Various observations suggest that the mitosis-stimulating agent is accompanied by a mitosis-inhibiting factor.

4. In comparison with other plant and animal cell division stimulating agents, the active agent in the orbital gland extracts most nearly resembles agents occurring in embryonic animal tissue, viz. the induction agent and the growth-promoting substances occurring in embryonic extracts which stimulate the growth of tissue cultures. The agent also has properties in common with the cancer virus agent. auxin, botanical wound hormones and with the osteogenic factor in bone tissues extracts which induces pluripotent connective tissue cells to metaplastic bone formation.

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CYTOLOGICAL STUDIES OF SARCOMA 37

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INTRODUCTION

For the past seven years we have been using Sarcoma 37 as one of the test materials for initial screening of chemotherapeutic agents applied to mouse tumors. It is similarly employed by the Division of Chemotherapy of the National Cancer Institute, from which our tumor stocks were originally obtained through the courtesy of Dr. M. J. Shear. During these years we have received many requests for supplies of transplantable tumors to be used in connection with experimental study, particularly from laboratories that are not primarily concerned with the maintenance of tumor stocks. Numerous inquiries have come to us from investigators in such laboratories concerning the origin of the tumor, the type of tumor, and other matters pertaining to its growth and characteristics. In the belief that our observations on the morphology and behavior of control tumors, made over a period of years, may be of assistance to other investigators, some of the data are herewith recorded.

ORIGIN OF THE TUMOR

Sarcoma 37 was developed at the Imperial Cancer Research Fund Laboratories in England about forty years ago. The account of the establishment of the tumor strain was reported by Bashford (3), whose original strain was an adenocarcinoma of the mouse mammary gland. The primary tumor contained acinous and alveolar areas. Two tumors were derived from the primary transplantation from which separate strains were propagated. The descendants of these two strains passed through a mixed tumor stage which eventually resulted in pure sarcoma at the 22nd generation. The predominating cell type is an undifferentiated spindle cell (see Fig. 1).

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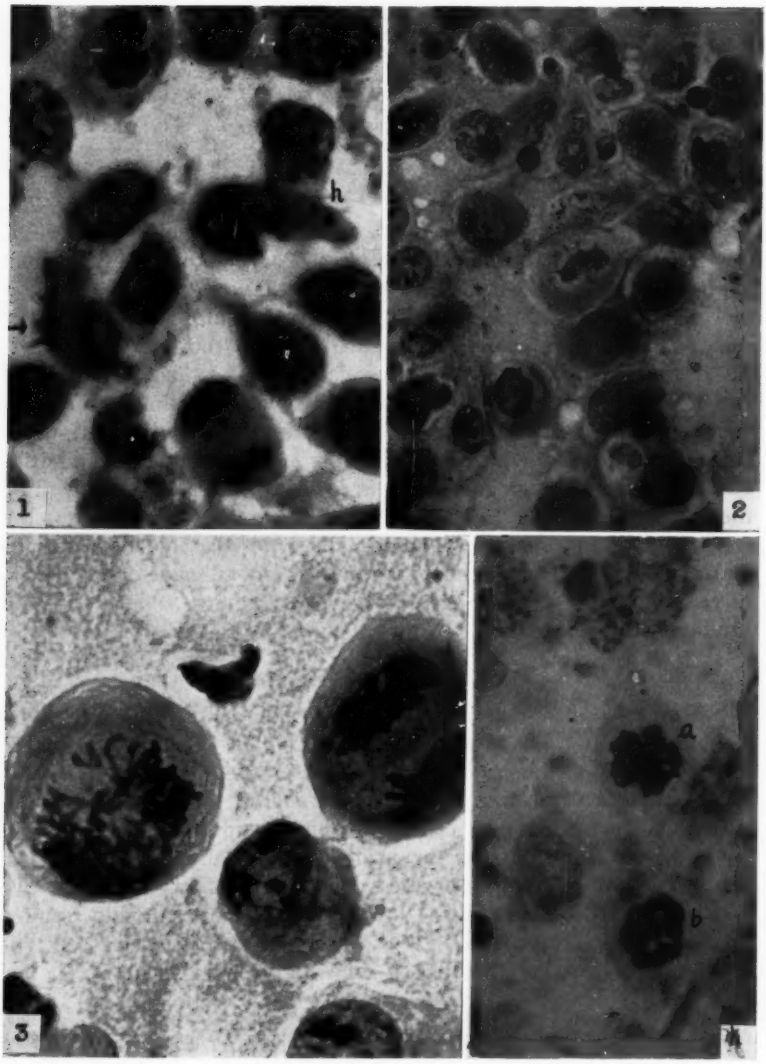
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Explanation of Plates

PLATE I

Fig. 1. Acetic-orcein smear of Sarcoma 37, showing general cell type and normal mitosis. In the telophase figure, note two granules, surrounded by faintly stained halos, slightly above the central axis of the spindle. (See also Figure 5.) At *h*, a larger body, surrounded by a halo, appears in the cytoplasm. 440×.

Fig. 2. A Flemming tri-color smear of S-37, showing spindle fibers in the lateral view of a metaphase figure; also darkly stained spheres of various sizes (lipid degeneration products). 320×

Fig. 3. Smear preparation of S-37 stained with Kull's stain following Benda fixation, showing delicate threads linking one of the chromosomes to the spindle. The late prophase nucleus on lower margin of the photograph shows two normal nucleoli; the nucleus above has several large, confluent ones, like those described on p. 119. 990×

Fig. 4. Acetic-orcein smear of S-37, showing two types of metaphase configuration, both of which occur commonly in this tumor. 440×

Techniques

Implantation. Routinely fragments of the tumor are introduced subcutaneously in the axillary region by means of a 12-gauge trocar. At times when the tumor does not appear to be growing well we have found it useful to implant with a smaller trocar into the leg muscle, or, following a method devised at the National Cancer Institute, a tumor brei suspended in saline solution can be injected hypodermically. The latter method is more satisfactory for introduction into solid tissue, such as leg muscle, than it is for subcutaneous injection, which results in a more diffused tumor.

Staining. In the course of our studies, many different types of fixation and staining have been employed. A useful general technique for the study of whole cells is the acetic-orcein smear technique described by Diller (5). This gives essentially the same staining effect as the Feulgen technique without the tedious steps involved in hydrolysis. The chromosomes appear magenta against a pale green background; the nucleoli are orcein-positive in interkinesis, but during prophase take up only the counterstain. For more precise chromatin estimation, however, we employ the Feulgen technique, either with smears or sections. Flemming fixation followed by Flemming's tri-color is particularly good for the study of mitochondria, or for the evaluation of lipid degeneration products (Fig. 2), since these are osmiophilic. Another osmic technique which demonstrates at one and the same time chromosome threads, spindle fibers and cytoplasmic components, is the modification of Benda-Kull (Fig. 3) devised by Schrader (18). Even such delicate structures as the fibers connecting the centromere to the spindle are plainly differentiated (Fig. 3). Other useful techniques for cytological study of these tumors were described by Ludford (13).

For the study of inclusions which do not appear to be normal components of the cell, we apply either to smears or sections the Robinow technique (17) for the study of bacterial nuclei; the McManus technique for demonstration of carbohydrates, as modified by Kligman and Mescon (12); and the Protargol technique of Moskowitz (16). In the case of the Robinow preparations, we frequently make use of "tissue imprints" which enable us to study the dissociated or loosely associated cells, such as blood cells and free tumor cells, without the distortion due to the pressure exerted in smearing. Each of these

techniques reveals different components and different inclusions in the cells, and together they have given us much information concerning response of different parts of the cell to chemotherapeutic agents.

Vascularization and Growth Patterns

A microscopic study of the growth of implanted fragments introduced subcutaneously by trocar was made at 24-hour intervals. Blood supply reaches these fragments within 24 hours after implantation in almost all cases. Macroscopic observations indicate that the portion which is in contact with the blood supply becomes immediately translucent; where there is no blood supply the implant does not attach and there is fragmentation of nuclei accompanied by coagulation of the cytoplasm. At the periphery, where the translucent tissue is to be found, one can observe healthy cells, though at 24 hours they are usually not in mitosis. From 24 to 42 hours after implantation, those fragments in contact with capillaries show cells in division mixed with degenerating cells. By the third day the blood supply is well established in the interior of the tumor, as has been shown also by Algire (*2*); in our experience, if capillaries do not reach the tissue by this time, the implant does not grow. In any event, the central portion of the implant usually degenerates and remains up to ten days after implantation as a yellowish-white mass of dead tissue. On the fourth day, capillary branches penetrate deeply into the tumors and the entire tissue becomes involved in mitotic activity. During the period from 18 to 72 hours we observe large numbers of giant prophase nuclei which appear to go into division immediately when vascularization is established.

The most troublesome feature of the use of Sarcoma 37 in the evaluation of agents which exert a selectively destructive action on tumor cells is its occasional tendency to regress spontaneously. At times conditions for maintenance of the animal colony in our former laboratory buildings were not optimum, and the degree of spontaneous regression of tumors implanted in Swiss mice reared in our Institute occasionally reached 16% in females and 10% in males. At the same period, investigators at the National Cancer Institute reported a very low percentage of spontaneous sloughing of tumors implanted in hybrid C x A mice (CAF_1). This was true also of tumors implanted by us in inbred C3H mice, and in C3H x Swiss hybrids. There is little difference, in this respect, between heterozygous Swiss mice and

inbred "A" strain mice now being reared in the main colony of our Institute—both can slough some of the tumors spontaneously after two or three weeks of growth, though the percentages are low and not constant. Sex differences are noticeable not only in the incidence of spontaneous regression, but also in the higher percentage of response of tumors borne by females to some chemotherapeutic agents. In addition, there are individual differences between hosts, since fragments of the same tumor implanted into litter mates of the same sex do not behave identically with respect to spontaneous regression.

There is also an observable seasonal deviation with regard to spontaneous sloughing. At times in the heat of the Philadelphia summer, our tumors did not grow well, or were resorbed spontaneously in the percentage indicated. In our new laboratory, where our experimental colony is maintained at a constant temperature of 70-72°, these adverse occurrences are greatly reduced, though we still observe some slight tendency to seasonal drop in viability of the transplants.

Occasionally we have observed that tumors grow well for the first two weeks after implantation, sometimes attaining a length of as much as 25 mm., and then begin to slough spontaneously in the third week of growth. Partial sloughing of large tumors is probably the consequence of imperfect blood supply in some portions of the tumors. Nevertheless, in such instances the tumors continue to grow and kill the host in about 21 days.

The tendency of Sarcoma 37 and other transplanted tumors to regress spontaneously is undoubtedly at times related to bacterial infections of the host, since tumors that show feeble growth frequently yield *Salmonella* or other bacteria in culture. Probably other organisms which we have isolated from these tumors (Diller, 6) have their influence also on growth rate and/or suppression. During the past year we have followed the technique of Dr. Georg Klein who was recently a visitor in our Institute. Sarcoma 37 cells suspended in sterile saline are injected into the peritoneal cavity of a mouse. This results in the production of either an ascites tumor, or a small tumor mass, depending on the concentration of the cells (Klein, 10, 11). Such a small tumor arising after several transfers, can be used very successfully for solid mass transplantation, and the tumors resulting therefrom show, at least temporarily, little tendency toward spontaneous regression.

Growth rates are determined in our experiments by the formula of Beck (Diller, Beck *et al.*, 7):

$$\frac{1nV_2 - 1nV_1}{(t_2 - t_1)} = K$$

This is based on a rough calculation of volume, in which the length, width and thickness, as determined by caliper measurements, are used to determine the volume of a half spheroid:

$$V = \frac{1 \times w \times h}{0.524}$$

Frequently, growth curves based on this estimate are much more helpful than an overall measurement at the beginning and end of an experiment, since suppressive action of an agent, even though temporary, can be detected. The use of the ascites form of tumor for quantitative as well as qualitative estimation of tumor cell damage is discussed by Klein (10) and also by Goldie and Felix (8).

Nuclear Phenomena

Chromosome Conditions. These were described for Sarcoma 37 in 1930 by Ludford (15). The maximum number of chromosomes delineated by him in metaphase was 36 per nucleus. With the acetic orcein technique especially as applied to well-extended smears, and also with Benda-Kull preparations of cells deliberately teased apart, we have found metaphase plates in which a full complement of 40 chromosomes (the normal diploid number for mouse cells) can be counted. However, Hauschka and Levan (9) reported that in the ascites form of Sarcoma 37 the nuclei are typically tetraploid, and that there are also numerous aneuploids.

On the basis of counts from paraffin sections made over a period of seven years we have determined that the multipolar spindle configurations are predominately tripolar in solid tumors. Therefore, if the same chromosome conditions prevail in solid tumors as in the ascites form of Sarcoma 37 the nuclei are typically tetraploid, and would necessarily give rise to aneuploid complexes. These apparently arise in other ways also, e.g. by elimination of partial complexes as described below.

Polyploidy is common in young transplants of solid tumor. When capillary infiltration is accomplished, the polyploid complexes are

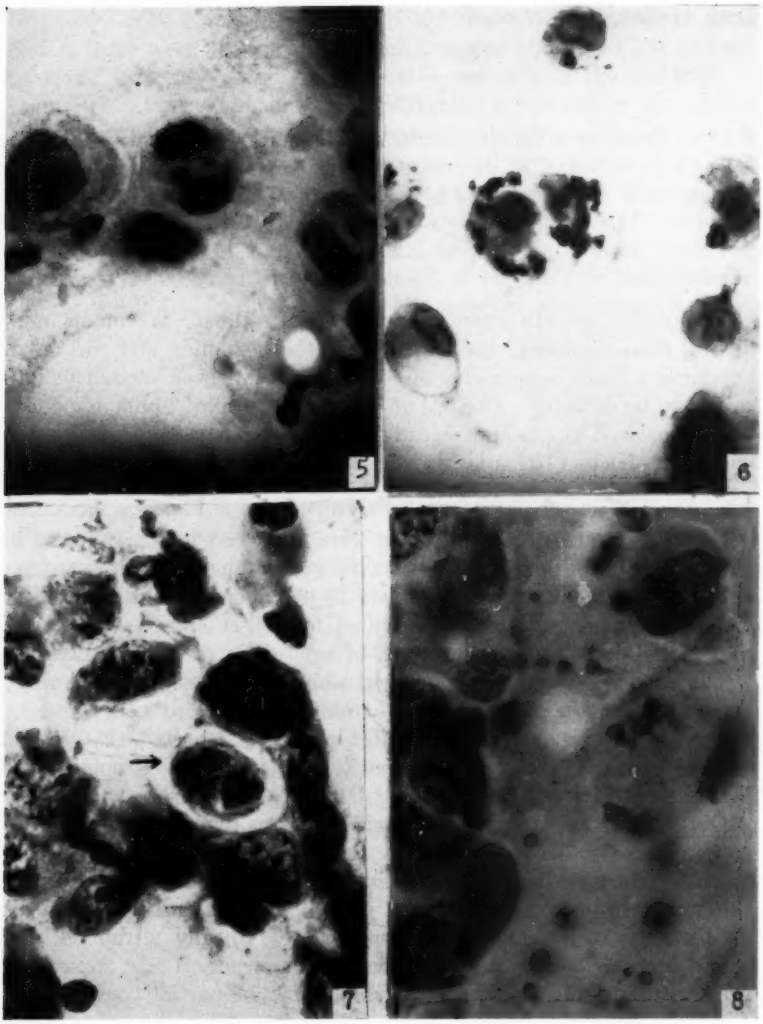
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PLATE II

FIGS. 5 AND 6. Photographs made by Dr. Miriam Flower of the Polaroid Corporation with the color-translating ultraviolet microscope. Magnification: 875×.

FIG. 5. Smear of S-37, fixed in Carnoy's fluid. No stain. Note body mid-way of spindle, clearly demonstrated at a wave-length of 263 Å.

FIG. 6. Cells of tissue culture of S-37, fixed in Zenker-formol. Note dense body in nucleus at 281 Å.

FIGS. 7 AND 8. Multipolar spindles in S-37.

FIG. 7. Flemming tri-color smear of S-37. Tri-polar spindle in an area of degeneration (note scattered osmiophilic masses). 440×

FIG. 8. Paraffin section of S-37 stained with orcein and fast green. Cell at tip of arrow is in late prophase and shows multiple groupings of chromosomes which should lead to multipolar spindle formation, or to asymmetric segregation without spindle formation. 440×

segregated according to several different patterns. There may be perfectly normal bipolar spindles giving rise to permanently polyploid nuclei; or multipolar spindles may be found as in Figures 7, 8 and 25. More rarely the polyploid configurations give rise to multinucleate cells. In general, however, during the first wave of mitotic activity post-implantation, there arise aberrant configurations which suggest that supernumerary chromosomes are being eliminated from the spindle. Some examples appear in Figures 21, 22 and 23, from paraffin sections stained in acetic orcein and fast green. These eliminations are accomplished either by multiple spindle formation, or by pinching off the supernumerary chromosomes in a bud, as in Figure 23.

Two types of metaphase figure are also frequently observed. In one the chromosomes remain slender and discrete (Fig. 4*a*) and in the other they are clumped and thickened (4*b*). Attention is called to this phenomenon because in early stages of our studies we had assumed the phenomenon shown in Figure 4*b* to result from response to a chemical agent; however, further study has shown this to be common in control tumors, not only in the solid form but in the free cells of S-37 ascitic fluid. This suggests a condition formerly attributed by Biesele (4) to "polyteny", i.e. multiplication of component chromosome strands without separation at mitosis.

Nuclear Inclusions. Frequently in late anaphase or early telophase an unidentified body is found midway of the spindle. This is shown by acetic orcein staining in Figure 1, and also appears in a photograph made by the Polaroid Corporation* with their color-translating ultraviolet microscope at a wave length of 263 Å (Fig. 5) as a light green spherule. Another unexplained body which is not visible in the light microscope but which appears in the ultraviolet pictures is shown in Figure 6. This consists of a dense intranuclear mass almost half the size of the nucleus which is demonstrated most clearly at a wavelength of 281 Å and appears in color-translation as a blue body.

Nucleoli. During the "resting" phase before the onset of waves of mitosis post-implantation, giant nuclei, presumably resulting from polyploid complexes, are observed. At such times the normal number of two nucleoli is increased to four, or even many more. Numbers of nucleoli were used by Biesele (4) as markers for the number of chromatin threads present in the nucleus of the cancer cell, and up

* We are particularly indebted to Dr. Miriam Flower of the Polaroid Corporation for making these studies.

to a certain point this is a very useful index; but in the case of giant nuclei, the greatly distended prophase nucleoli show a tendency to flow together, forming large irregular masses which are not Feulgen-positive. In such confluent masses it is impossible to recognize the individual elements or to ascertain their number.

Cell "Inclusions"

Apart from the expected cytoplasmic bodies such as the mitochondria and Golgi apparatus, which can be demonstrated following osmic fixation with a number of stains (Ludford, 13) we have found, in a great proportion of the cells, bodies which do not correspond to any known cellular component. Elsewhere (Diller, 6) we have attempted to relate these bodies to micro-organisms that can be isolated from the tumor on appropriate culture media, and the types of inclusion that can be demonstrated by the Robinow, Protargol and Benda-Kull techniques appear in Plate V of that earlier paper. (See also Figs. 15, 16, 17, 19 and 20 of the present article). Similar inclusions can be demonstrated by means of the Papanicolaou technique for the study of malignant smears. These bodies are of varying size. Some appear to be budding, others are attached to filaments. A common configuration is that of a minute granule surrounded by a halo, in which the central portion can be demonstrated either by the Robinow stain or by the Feulgen technique (Figs. 9, 10), although the latter is usually very faint and can be observed only at high magnifications. These Robinow-positive and Feulgen-positive granules have also been demonstrated inside the cells of other tumors by Wuerthele-Caspe (20), using a modification of the Ziehl-Neelsen technique devised by Alexander-Jackson (1), as having acid-fast properties. When prepared by the McManus technique, the "halo" is shown to be delimited by a McManus-positive, therefore polysaccharide-bearing, outer rim.

Types of Cellular Degeneration

The quantitative evaluation of the number of cells destroyed or inhibited by therapeutic agents presents many difficulties in solid tumors, because it is found that most tumors show some degenerating cells at all times, even when they have not been experimentally treated. At the center of the tumor there may be degenerating cells which are part of the residual implant and areas of necrosis which are presumably the consequence of imperfect blood supply; but, in addition,

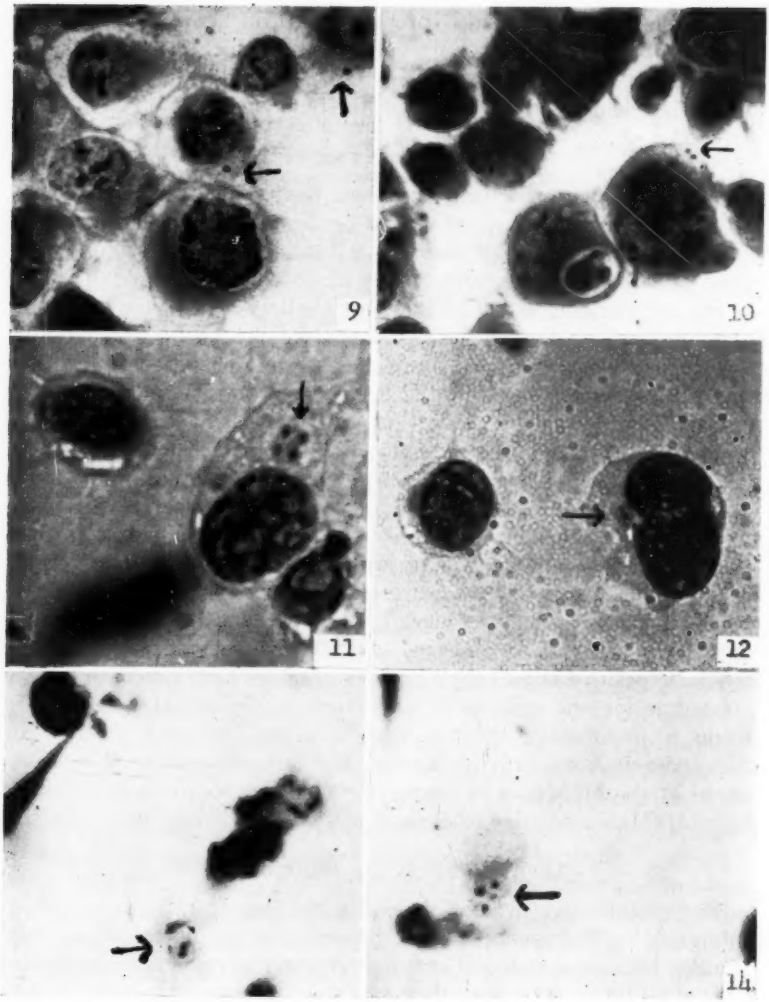
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PLATE III

FIG. 9. Smear preparation of Sarcoma 37, fixed in Carnoy, stained with Feulgen-fast-green. Note inclusion bodies, surrounded by halos, at tips of arrows. Central granule is Feulgen-positive. 440×

FIG. 10. Zenker-Robinow preparation of Sarcoma 37 .tissue imprint. Yeast-like inclusion body at "y", minute granular inclusions surrounded by halos at tips of arrows. Note that one of these is bipartite, as though it may have divided. 440×

FIG. 11. Carnoy-Feulgen preparation of smear of Sarcoma 37 ascites fluid. At tip of arrow, note inclusion body containing 4 small granules (central granule divided twice?). 900×

FIG. 12. Smear of Sarcoma 37 ascites tumor, fixed in Zenker, stained with McManus, counterstained with Harris' hematoxylin. McManus-positive inclusion body in cytoplasm. 900×

FIG. 13. Tissue culture of Sarcoma 37 originally prepared by Benda-Kull (osmic fixation), decolored and restained by the McManus technique. Bi-partite McManus-positive inclusion body, tumor cells unstained. 900×

FIG. 14. The same. Quadripartite McManus-positive inclusion body near unstained tumor cell nucleus. 900×

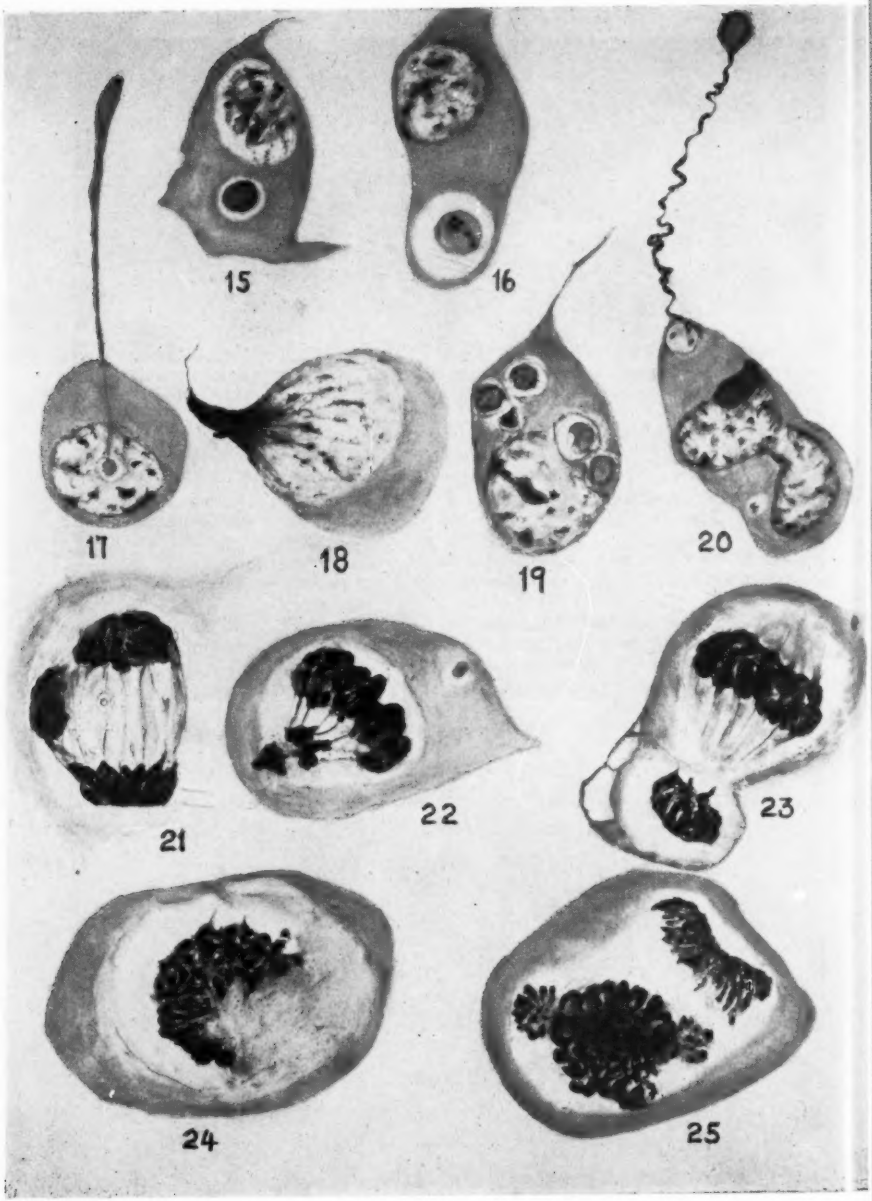
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PLATE IV

FIGURES 15-20. Sarcoma 37 smear preparations, fixed and stained by the Benda-Kull technique. 1500×

FIGS. 15, 16, 17, 19 and 20 show inclusion bodies, which in Figures 17 and 20 are associated with filaments extending beyond the cell body.

FIG. 18. Degenerating nucleus showing pycnotic polar mass.

FIGURES 21-25. Sarcoma 37, fixed in acetic-alcohol, sectioned in paraffin and stained with orcein-fast-green.

FIG. 21. Tri-polar massing of chromosomes in a configuration that suggests that the lateral mass will be eliminated in cytokinesis.

FIG. 22. Precocious movement of chromosomes to one pole of spindle. Probably a variant of the same process illustrated in Figure 23.

FIG. 23. Normal metaphase spindle associated with additional chromatic mass which is apparently being pinched off at one pole.

FIG. 24. A common configuration in degenerative areas of Sarcoma 37. Metaphase chromosomes appear to mass at one side of the nucleus without spindle formation. This is similar to Ludford's figures of cells which he believes are incapable of further development.

FIG. 25. Multipolar spindle: the mass lying at the lower level represents a normal metaphase spindle in lateral view; two other groups of chromosomes are already in telophase and oriented obliquely so that the mass superimposed on the metaphase grouping is in polar, the remaining group in lateral, view.

there may be other areas of "spot necrosis" throughout the tumor, whether as the result of capillary lack or failure, of infections, or of aberrant mitotic procedures. The commonest type of "spontaneous" degeneration is pycnosis, a rounding up and shrinkage of the cell leading eventually to fragmentation into granular and spherical bits of debris. The intermediate changes leading to degeneration, however, may take numerous forms, an example of which appears in Figure 24. This apparently arises from an asymmetric distribution of chromosomes in mitosis, which may assume various aspects. Similar phenomena were described by Ludford (14) in Sarcoma 37 (his figures 4-8) and ascribed by him to lack of spindle formation. He believed such cells to be incapable of further development.

Another peculiar, but common, phenomenon in areas of spontaneous degeneration is that shown in preparations made by the Benda-Kull technique, which demonstrates extensive formation of filaments which are not extruded chromatin, since they do not stain with chromatin dyes. The entire nucleus is frequently drawn up to one side of the cell and twisted into a mass (Fig. 18) and at times an osmiophilic filament is seen to proceed from the tip of the chromatic cap. Sometimes one has the impression that the filament has twisted about a portion of the nucleus and has drawn the chromatin into a contorted structure at one pole of the cell; or the filaments may be attached to inclusion bodies (Figs. 17 and 20), as was shown also in Figure 22 of an earlier paper (6). The question of whether the inclusion bodies arise endogenously or exogenously, and if exogenously, whether by invasion or phagocytosis, is currently under investigation in vivo and in vitro in connection with a number of ascites tumors.

Summary

Cytological conditions obtaining in untreated mouse Sarcoma 37, which is used in the Institute for Cancer Research in Philadelphia and elsewhere as a test material for preliminary chemotherapeutic screening tests, are described. Mitotic abnormalities, aberrant behavior of chromosomes and cytoplasmic components, spontaneous regression, and types of spontaneous cellular degeneration, are discussed, together with a description of techniques useful in determining the structural changes induced by chemotherapeutic agents in different parts of the cell.

Cellular abnormalities are here set down for the benefit of those who are not experienced in the study of this particular tumor, and

who could be misled into thinking that some of the aberrations are the result of experimental manipulations. We believe that it would be helpful if other investigators would prepare similar reports on the tumors which they employ most extensively in experimentation. Such reports might serve as the basis for the eventual compilation of an atlas of experimental tumors.

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SIGNIFICANCE OF CALORIC INTAKE AND SPECIFIC EFFECTS OF HIGH-FAT DIETS ON SKELETAL GROWTH AND DEVELOPMENT OF MICE*

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Male mice of strain C57 black fed from weaning on a diet containing 29% fat showed accelerated epiphyseal growth and development as compared with mice given a stock diet of Purina Laboratory Chow containing 5% fat (5). The results obtained in these qualitative feeding experiments might have been due to differences in the caloric values of the diets, or they might have been due to specific effects of the fat. In order to decide this question, quantitative experiments were carried out, the results of which are presently reported. These investigations involved the feeding of rations equal in calories but differing in composition of the basic nutrients furnishing the calories.

MATERIAL AND METHODS

Seventy male mice of the closely inbred strain C57 black, four to five weeks old, were divided into five groups, hereafter referred to as groups I to V. These animals were fed three different diets composed of a common base with an isocaloric supplement high in protein, fat, or carbohydrate respectively. The composition of the diets** was, as follows:

	Grams	Calories
Base		
Lactalbumin	50.0	215
Cornstarch[1]	20.0	84
Lard[2]	5.0	47

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** We are indebted to Dr. C. M. McCay, Professor of Nutrition, Cornell University, Ithaca, N. Y., for his advice concerning the composition of these rations.

	Grams	Calories
Base (continued)		
Alphacel	9.0	0
Salt Mixture U.S.P. No. 2	12.5	0
Brewers Yeast dried	20.0	79
Dried Liver[3]	5.0	15
Cod Liver Oil	2.0	19
Wheat Germ Oil	1.5	15
Total	125.0	474
	1.0	contains 3.7
Diet A (*High-Protein*)		
Base	42.0	155
Cornstarch[1]	10.0	42
Lactalbumin	47.0	202
Total	99.0	399
	1.0	contains 4.0
Diet B (*High-Fat*)		
Base	42.0	155
Cornstarch[1]	10.0	42
Lard[2]	22.0	205
Total	74.0	402
	1.0	contains 5.4
Diet C (*High-Carbohydrate*)		
Base	42.0	155
Cornstarch[1]	58.0	244
Total	100.0	399
	1.0	contains 4.0

Unless mentioned otherwise, all nutrients were secured from the Nutritional Biochemical Corporation, Cleveland, Ohio.

[1] Topmost Brand.

[2] Swift's Silverleaf Brand.

[3] We are indebted to the Armour & Co., Chicago, Illinois, for the generous supply of dried liver powder.

In preliminary experiments, mice kept on the high-protein ration were found, as a rule, to have the lowest caloric intake. Therefore, in each experimental set, the animal fed the high-protein diet ad libitum was used as the pilot animal. This group will be referred to as group I. Group II comprises the animals that were offered the

high-fat diet in amounts restricted to the caloric intake of their respective pilots, while mice of group IV were offered the high-carbohydrate diet in correspondingly restricted amounts. Thus, animals of groups I, II, and IV were offered isocaloric quantities of rations differing in composition of protein, fat, or carbohydrate respectively; animals of group III were fed the high-fat diet, and those of group V were given the high-carbohydrate diet ad libitum. Water was available to all animals at all times. Groups of mice were sacrificed, when the animals were 2, 3, 4, or 6 months of age.

In the beginning, two mice were kept together in one cage. Subsequently it was decided to cage the mice individually, because some animals developed aggressive traits and prevented the other animal in the cage from getting its share of food.

In order to determine as accurately as possible the amount of food consumed, the wastage of food by the animals had to be kept at a minimum. Therefore, the rations were offered in the form of cookies placed in cube-shaped containers made of 3″ wire mesh with a lid that could easily be opened and closed every time the food was given. In order to prevent spilling of the food, the containers were suspended from the top of the cage so as to touch the bottom of a round metal disk 2½″ in diameter and ½″ high. Broken off pieces of food would drop into this dish and could be recovered, when the leftovers were weighed. As a second precaution, the amount of food offered to the mice fed ad libitum was kept as small as possible, just enough to have about 10 to 20% of the total left over. If larger amounts were at the animal's disposal, wastage was increased. The food intake of each mouse was determined daily except Sundays and holidays, when double rations were given. The animals on restricted rations usually consumed their allotments; occasionally small amounts were left over.

The mice were weighed once a week. At the end of the period of observation, the animals were sacrificed, and the kneejoints including the lower end of the femur and the upper end of the tibia were removed. Bouin's solution served as a fixative as well as a decalcifying solution. The tissues were embedded in paraffin, sectioned semiserially at 5μ, and stained routinely with hematoxylin and eosin; some sections were stained with Best's carmine or osmic acid, light-green and fuchsin, for the demonstration of glycogen or fat respectively.

The width of the growth zones was measured with an object micrometer (0.01 mm. Carl Zeiss, Jena), averages being established

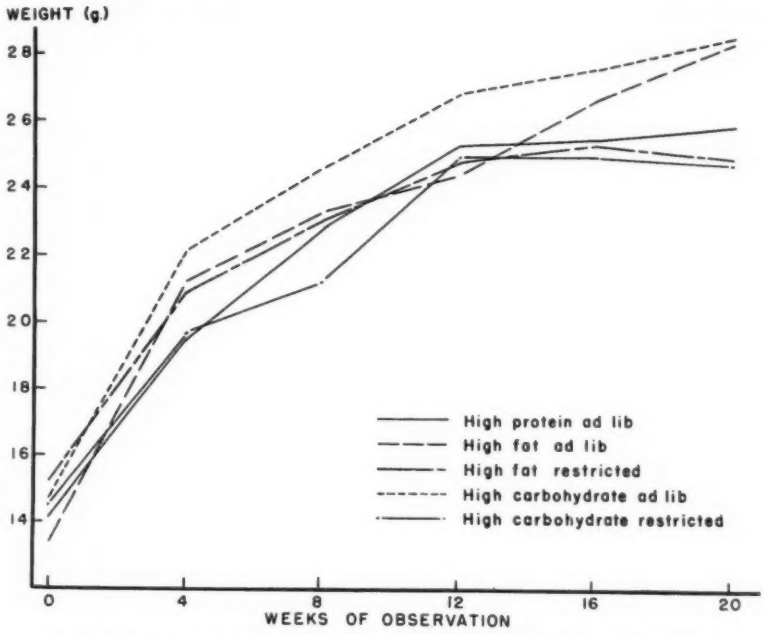
from different sections. The cells in the individual cartilage cell rows were counted, and the proportion of columnar cells to those of the hypertrophic type was determined. The degree of hyalinization of the cartilage, the breakdown of the cartilage as indicated by the formation of amorphous "plugs", the resorption of the primary spongiosa, and the presence or absence of a subepiphyseal plate delimiting the epiphyseal cartilage from the metaphysis served as additional criteria of epiphyseal development and ageing.

Observations

The diets were readily taken by the animals and were well tolerated. The food intake of the mice offered restricted amounts varied somewhat. A number of these animals almost always consumed their entire allotment; some did so on many but not on all days, and still others left fractions of a gram over on most days, even though they consumed less than their mate fed the same diet ad libitum. Since the amount of left-over food was registered to one-tenth of a gram, the actual amount of food consumed could easily be determined. The fact that the intake was sometimes below that of the pilot animal did not interfere with the expected results, since for the purpose of the present experiments it was essential only that these animals did not consume more calories than the pilots.

Towards the end of the growth period, a number of animals became infested with mites which caused skin irritations and which it was impossible to get rid of. In accordance with observations in other laboratories, we attribute this to the fact that animals on synthetic or calorically inadequate diets usually are less resistant to such infestations than animals on natural diet (2-4, 6).

Gain in weight was comparable to that of animals kept on Purina Laboratory Chow. The weights of the animals are given in Graph I. Animals kept on the high-carbohydrate diet ad libitum showed the fastest gain in weight, with those kept on the high-fat diet ultimately reaching the same weights. Between four and ten weeks of observation, the weights of the animals on the restricted high-fat regimen tended to exceed that of the pilots, while the weights of animals on the restricted high-carbohydrate diet tended to remain slightly below that of the pilots. From the fourth month of observation on, the weights of the animals fed the high-protein ration ad libitum and of those receiving the high-fat or high-carbohydrate ration in amounts restricted to the caloric intake of the pilot animals were similar.



GRAPH SHOWING THE MEAN WEIGHTS OF ALL ANIMALS FED THE VARIOUS DIETS

The values for the mean caloric intake of all animals are given in Table 1. There was on all diets a steady decline in caloric intake per

TABLE 1
MEAN DAILY CALORIC INTAKE OF ALL ANIMALS FED AD LIBITUM

	Weeks of observation									
	2	4	6	8	10	12	14	16	18	20
				I. Total						
High-Protein diet	10.0	10.9	11.2	11.3	11.2	12.1	12.1	11.9	11.2	11.7
High-Fat diet	12.5	11.7	12.3	12.1	12.1	11.9	10.9	9.7	11.0	11.1
High-Carbohydrate diet	11.8	11.6	12.4	12.1	11.8	11.8	10.9	10.4	11.5	10.8
				II. Per Gram Body Weight						
High-Protein diet	0.57	0.57	0.56	0.50	0.48	0.48	0.49	0.49	0.44	0.45
High-Fat diet	0.66	0.55	0.53	0.52	0.51	0.49	0.42	0.36	0.40	0.39
High-Carbohydrate diet	0.59	0.53	0.52	0.49	0.44	0.41	0.40	0.38	0.40	0.38

gram body weight from the second week towards the end of the period of observation. Initially, the caloric intake of the mice fed the high-fat diet ad libitum was higher than that of the high-protein fed animals; however in the end, the caloric intake of animals fed the high-fat diet declined to a lower level than that of the mice fed the high-protein diet. The caloric intake of mice kept on the high-carbohydrate ration ad libitum resembled at first that of the animals receiving the high-protein diet, but from the eighth week of observation on, it declined more rapidly than that of the latter; it finally reached the same level as that observed in the mice fed the high-fat diet ad libitum.

Microscopic Examination

In a general way, the differences observed in the various experimental groups were quantitative in nature or concerned the time of appearance of changes. A few findings seemed to be common to animals receiving the same diets. The growth zones of animals kept on the high-protein diet showed comparatively few cells of hypertrophic type, and the metaphyseal spicules were delicate. Animals fed the high-carbohydrate diet developed large hypertrophic cells, and the metaphyseal spicules contained abundant bony substance. The growth zones of animals consuming the high-fat diet could usually be recognized by the early and conspicuous degeneration of cartilage.

The main findings in the growth zones obtained after 1, 2, 3, or 5 months of observation are presented in Tables 2 to 5. Individuals belonging to the same set are grouped together. In order to facilitate tabulation, the following gradation of the findings was adopted:

Hyalinization: "Trace" denotes the finding of an occasional small wedge of hyaline material between the cartilage cell rows, "+" indicates the encroachment of moderate amounts of hyaline matrix upon the cartilage, "marked" hyalinization is associated with a considerable decrease in the number of cartilage cells.

Plug formation: "Trace" indicates the beginning formation of plugs of degenerated cartilage, "+" denotes the occurrence of one or of several small such plugs, "marked" plug formation is characterized by the presence of numerous small or thick plugs.

Subepiphyseal plate: An "early" plate is composed of a discontinuous layer of bone with linking of the distal parts of the primary

spicules; "+" means the presence of a continuous bony plate; "marked" indicates the presence of a continuous thick plate.

Articular cartilage: The grading of the articular age changes has been fully described elsewhere (5).

By the use of these criteria, the age order of the animals within each experimental set was established, and the lowest number was given to the animal with the youngest looking growth zone. Some of the characteristic changes are illustrated in photomicrographs 1 to 5.

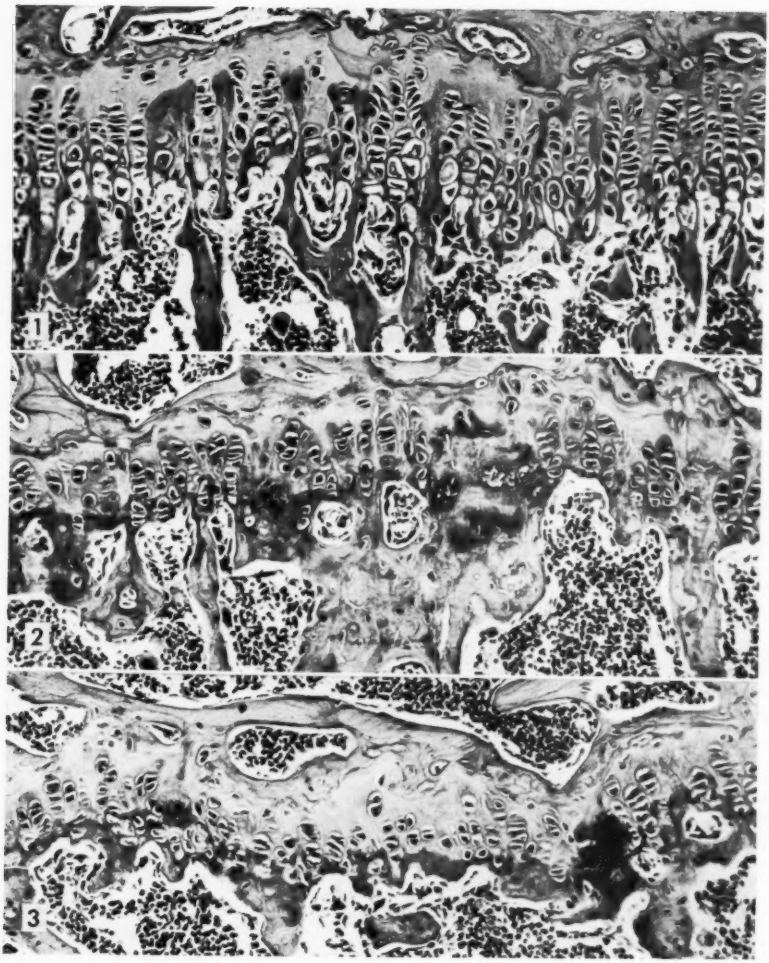
Findings at the age of 2 months: (Table 2).

In all three sets, the growth zones of the mice fed the high-protein diet (9, 14, 64) showed the most youthful appearance. Mice receiving the high-carbohydrate diet in restricted amounts (12, 17, 67) were close seconds in physiologic age order. In sets one and two, the third places were taken by the mice fed the high-carbohydrate diet ad libitum (13, 18); in set three, the third place was occupied by mouse 65 which had consumed the high-fat diet in restricted amounts. Two mice fed restricted quantities of the high-fat diet (10, 15) and one mouse (68) kept on the high-carbohydrate diet ad libitum occupied the fourth places in physiologic age order. The growth zones of the mice given the high-fat diet ad libitum (11, 16, 66) disclosed the most advanced state of epiphyseal development and ageing.

Some details observed in this group of animals seem noteworthy: In the first set, mouse 13 had consumed 337 calories, that is more than any other animal of this set; yet the growth zone of this mouse looked more youthful than that of mice 10 and 11 which had eaten 266 or 286 calories of the high-fat respectively. In the second set, the growth zone of mouse 15 appeared older than that of mouse 18. The former animal had consumed 248 calories of the high-fat diet; the latter had eaten 357 calories of the high-carbohydrate ration. In the third set, the growth zone of mouse 66 with a consumption of 311 calories of the high-fat diet disclosed the most advanced degree of development, while mouse 64 with an intake of 333 calories of the high-protein diet showed the most youthful appearing growth zone of the entire set.

Findings at the age of 3 months: (Table 3).

Mice fed the high-protein diet (21, 51, 69) had the most youthful looking growth zones: Growth and development of the cartilage were in good progress, and regressive changes were slight. Two mice fed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Explanation of Photomicrographs

Sections through the proximal growth zones of the tibia of male mice of strain C57 black, 4 months of age. Magnification 200×

Fig. 1. High-protein diet ad libitum: Cartilage cell columns well developed, a few areas of hyalinization of the matrix between the cartilage cell rows are seen. The bony spicules are delicate and show some transverse linking.

Fig. 2. High-fat diet in amounts restricted to the caloric intake of the animal shown in Fig. 1: The growth zone is narrowed, the cell columns are shortened, hypertrophic cartilage cells are almost absent. Thick plugs of degenerated cartilage are seen to replace columns of cartilage cells. The subepiphyseal spicules have been replaced by a thick bony plate.

Fig. 3. High-fat diet ad libitum: The growth zone is markedly narrowed and poor in cells. Two plugs of degenerated cartilage are seen. There is a continuous subepiphyseal bony plate.

FIG. 4. High-carbohydrate diet in amounts restricted to the caloric intake of the animal shown in Fig. 1: The cartilage cell columns are well preserved; there is slight hyalinization of the matrix. Bone formation about the primary spongiosa is in good progress.

FIG. 5. High-carbohydrate diet ad libitum: The growth zone is inactive and contains plugs of degenerated cartilage. The subepiphyseal bony plate is continuous.

TABLE 2

WEIGHTS, CALORIC INTAKE, AND MICROSCOPIC FINDINGS IN THE GROWTH ZONES OF MICE

Age: 2 months

Type of diet	Mouse No.	Initial weight gm.	Maximum weight gm.	Total caloric intake	Width of growth zone (μ)	Epiphyseal cartilage				Microscopic age order within each set
						No. of cells columnar: hypertrophic	Hyalinization	Plug formation	Subchondral bony plate	
High-Prot. ad lib.	E 9	16.8	20.8	288	135	6-8:1-2	trace	trace	0	1
High-Fat restr.	E10	17.3	21.0	266	125	5-6:1	+	+	early	4
High-Fat ad lib.	E11	17.4	22.8	286	120	5-6:0-1	marked	+	early	5
High-Carboh. restr.	E12	17.5	22.2	281	130	6-8:1-2	trace	+	0	2
High-Carboh. ad lib.	E13	17.6	23.0	337	125	5-7:1-3	+	+	0	3
High-Prot. ad lib.	E14	16.1	20.8	298	140	5-7:2-3	trace	0	0	1
High-Fat restr.	E15	15.6	22.1	248	120	5-6:1-2	+	+	early	4
High-Fat ad lib.	E16	14.2	21.4	372	120	4-7:0-1	marked	+	early	5
High-Carboh. restr.	E17	14.5	21.0	283	135	5-8:1-2	+	0	0	2
High-Carboh. ad lib.	E18	14.5	23.2	357	125	5-7:1-2	+	+	0	3
High-Prot. ad lib.	E64	14.6	20.0	333	140	7-8:2-3	trace	0	0	1
High-Fat restr.	E65	13.8	20.2	282	130	5-7:1-2	+	0	0	3
High-Fat ad lib.	E66	13.0	19.2	311	100	4-6:0-2	marked	+	0	5
High-Carboh. restr.	E67	10.0	19.1	301	130	6-7:2-3	trace	0	0	2
High Carboh. ad lib.	E68	10.0	20.0	322	105	5-7:2-3	+	+	0	4

TABLE 3

WEIGHTS, CALORIC INTAKE, AND MICROSCOPIC FINDINGS IN THE GROWTH ZONES OF MICE

Age: 3 months

Type of diet	Mouse No.	Initial weight gm.	Maximum weight gm.	Total caloric intake	Width of growth zone (μ)	Epiphyseal cartilage				Microscopic age order within each set
						No. of cells columnar: hypertrophic	Hyalinization	Plug formation	Subchondral bony plate	
High-Prot. ad lib.	E21	17.7	21.0	557	105	5-6:2-3	+	+	early	1
High-Fat restr.	E22	18.7	23.5	497	90	4-5:0-1	marked	marked	marked	4
High-Fat ad lib.	E23	18.5	24.0	578	80 irreg.	3-5:0-1	marked	marked	marked	5
High-Carboh. restr.	E24	17.9	24.0	533	95	4-6:0-1	marked	+	+	3
High-Carboh. ad lib.	E25	17.8	24.0	584	100 irreg.	5-7:0-2	+	+	early	2
High-Prot. ad lib.	E51	12.3	17.4	442	100	5-7:1	trace	trace	early	1
High-Fat restr.	E52	15.2	21.1	395	90 irreg.	4-6:0	+	+	+	3
High-Fat ad lib.	E53	16.1	25.7	575	80 irreg.	3-5:0	+	+	marked	5
High-Carboh. restr.	E54	11.8	21.2	405	90	5-6:1-2	+	+	+	2
High-Carboh. ad lib.	E55	15.0	23.9	598	80	4-5:0-1	marked	+	+	4
High-Prot. ad lib.	E69	13.9	22.9	739	105	6-8:1-2	+	+	early	1
High-Fat restr.	E70	15.7	24.8	701	80 irreg.	4-6:0-1	+	+	+	3
High-Fat ad lib.	E71	15.7	24.7	872	65 irreg.	4-6:0	marked	+	marked	5
High-Carboh. restr.	E72	12.6	21.9	651	95 irreg.	5-6:0-1	marked	+	+	2
High-Carboh. ad lib.	E73	12.5	22.8	834	70	4-6:0-1	marked	+	+	4

the high-carbohydrate diet in restricted amounts (54, 72), and one mouse fed the high-carbohydrate diet ad libitum (25) took the second places in order of development. Third in line were mouse 24 which had received restricted amounts of the high-carbohydrate diet, and mice 52 and 70 fed the high-fat diet in restricted amounts. The fourth places in the order of age were occupied by mouse 22 kept on the high-fat diet in restricted quantities, and by mice 55 and 73 both of which had been fed the high-carbohydrate diet ad libitum. In the three mice fed the high-fat diet ad libitum (23, 53, 71), growth was about to cease. In all three sets, the mice fed the high-fat diet ad libitum were thus farthest advanced as far as epiphyseal development is concerned.

Some details of the findings presented in Table 3 may again be pointed out: In the mice of the first set, mouse 25 had consumed 584 calories of the high-carbohydrate diet; it took the second place in the order of development, whereas mouse 23 which had eaten almost the same amount of calories (578) of the high-fat diet occupied the fifth place in physiologic age order. In the second set, epiphyseal development of the mice with the highest caloric intake (mice 53 and 55; caloric intake: 575 or 598 respectively) was more advanced than in mice 51, 54, and 52 which had consumed 442, 405, or 395 calories of the three respective diets. Of the latter three animals, epiphyseal development of mouse 51 fed the high-protein diet was least and that of mouse 52 fed the high-fat diet was farthest advanced, although the caloric intake of the latter was about 12% less than that of the former. The same order was also observed in the corresponding animals of sets 1 and 3, in both of which the pilot animals had consumed more calories than the restricted animals, and still were retarded in development as compared to the latter. These findings demonstrate the role of the composition of the diet in their effects on skeletal growth. The role of the caloric intake on the other hand is indicated in set 3: The growth zones of mice 71 and 73 that had consumed 872 and 834 calories respectively looked older than those of mice 70 and 72 which had eaten 701 and 651 calories of the corresponding diets.

Findings at the age of 4 months: (Table 4).

In sets two and three, the growth zones of mice fed the high-protein diet (74, 81) again occupied the first, that is the least advanced position in physiologic age. Mouse 30 of the first set constituted the only

TABLE 4

WEIGHTS, CALORIC INTAKE, AND MICROSCOPIC FINDINGS IN THE GROWTH ZONES OF MICE

Age: 4 months

Type of diet	Mouse No.	Initial weight gm.	Maximum weight gm.	Total caloric intake	Width of growth zone (μ)	Epiphyseal cartilage				Microscopic age order within each set
						No. of cells columnar: hypertrophic	Hyalinization	Plug formation	Subchondral bony plate	
High-Prot. ad lib.	E30	13.3	17.6	672	130	6-9:1-2	+	+	0	2
High-Fat restr.	E27	14.0	26.5	505	90	3-6:0	marked	marked	marked	4
High-Fat ad lib.	E28	10.2	23.9	907	60 irreg.	2-6:0	marked	marked	marked	5
High-Carboh. restr.	E29	9.5	25.2	550	135	6-8:1-2	+	+	0	1
High-Carboh. ad lib.	E26	9.5	25.5	976	100	4-6:0	marked	+	+	3
High-Prot. ad lib.	E74	13.9	25.3	965	120	5-7:1-2	+	+	+	1
High-Fat restr.	E75	14.2	23.5	853	100	3-6:0	+	+	marked	3
High-Fat ad lib.	E76	14.3	22.5	1052	85 irreg.	3-7:0-1	marked	marked	+	4½
High-Carboh. restr.	E77	17.7	25.0	942	100	4-7:1	marked	+	+	2
High-Carboh. ad lib.	E78	18.7	25.6	958	85 irreg.	3-6:0	marked	+	+	4½
High-Prot. ad lib.	E81	13.4	29.2	995	100	3-7:0-2	marked	+	early	1
High-Fat restr.	E82	13.1	28.2	898	95	3-7:1	+	+	marked	3**
High-Fat ad lib.	E83	11.3	22.3	1042	60 irreg.	2-5:0	marked	marked	marked	5**
High-Carboh. restr.	E84	14.4	23.8	934	85	5-7:1	marked	+	marked	2
High-Carboh. ad lib.	E85	13.9	31.4	1188	70 irreg.	3-6:1	marked	+	early	4*

* Articular cartilage shows slight age changes (grade I).

** Articular cartilage shows moderately advanced age changes (grade II).

exception to the rule among 13 mice kept on this diet in that it occupied the second instead of the first place within the age order of the set. This might have been due to its comparatively high weight at the beginning of the experiment (13.3 gm.) as compared to that of mouse 29 which took the first position and whose initial weight had been 9.5 gm.; or, it might have resulted from the fact that mouse 29 was a poor eater which left over considerable quantities of its allotted ration. The animal consumed 122 calories less than the pilot, a difference of about 20%. In this connection it may be noteworthy that the animal receiving the high-fat diet in restricted quantities (mouse 27) consumed about 25% less than the pilot; but apparently, the high-fat contents of the ration more than made up for the low caloric intake, since the animal took fourth place in physiologic age order. This finding again points to the role of the composition of the diets in their effects on epiphyseal growth and ageing. In sets 2 and 3, the mice on the restricted high-carbohydrate diet (77, 84) took second place in epiphyseal development, as had been the case in five of six corresponding animals sacrificed at the ages of two or three months. Mice 75 and 82 fed the high-fat diet in restricted amounts, and mouse 26 fed the high-carbohydrate diet ad libitum held the third place in epiphyseal development. The comparatively young epiphyseal age of mouse 26 may be due to its low initial weight (9.5 gm.). Apparently, the high caloric intake of this animal (976 calories) did not suffice to compensate for the deficit in weight at the beginning of the experiment. The most advanced state of epiphyseal development was found in mice 78 and 85 fed the high-carbohydrate diet ad libitum, and in mice 28, 76, and 83 fed the high-fat diet ad libitum. In the second set, mice 74, 75, and 77 had consumed 965, 853, or 942 calories respectively; yet, the growth zone of mouse 75 with the lowest caloric intake but kept on the high-fat diet appeared older than that of the other two which had received 965 calories of the high-protein diet or 942 calories of the high-carbohydrate diet respectively. On the other hand in mouse 76, fed the high-fat diet ad libitum epiphyseal ageing was of about the same order as in mouse 78 fed the high-carbohydrate diet ad libitum, although the latter had consumed about 10% less in calories than the former. The advanced state of epiphyseal development of mouse 78 may again be related to its high initial weight (18.7 gm.). In the third set, mice 81, 82, and 84 which had an intake of 995, 898, or 934 calories respectively development

of the growth zones was most advanced in the animal kept on the high-fat diet (82), although its caloric intake was the lowest of the three animals. Moreover, in mouse 83 which had consumed 1042 calories of the high-fat diet, epiphyseal ageing was farther advanced than in mouse 85 which ate 1188 calories of the high-carbohydrate diet.

Slight articular age changes were present in mouse 85, and moderately advanced articular age changes were noted in mice 82 and 83.

Findings at the age of 6 months: (Table 5).

These are the sets in which some animals were kept in pairs within the same cage. The values for the caloric intake of these mice therefore represent the total consumption divided by two. Again in each of the three sets, the mice fed the high-protein diet (33, 42, 43, 56) showed the most youthful growth zones. Equally uniform results were obtained in the mice fed the high-fat diet ad libitum (36, 37, 45, 46, 59). The growth zones of the latter mice were the most aged ones within their respective sets; in particular, they appeared older than those of the mice receiving the high-fat diet in restricted amounts (34, 35, 44, 57, 58). Next in age to the group fed the high-protein diet were five of the six mice fed the high-carbohydrate diet in restricted amounts (38, 39, 47, 48, 61), and one mouse (63) fed the high-carbohydrate diet ad libitum. It is difficult to account for the retardation of epiphyseal development of this animal, especially since its final weight (30.4 gm.) was higher than that of its mate (28.5 gm.). Possibly this mouse may have had a lower caloric intake than appears from the mean value of 1351 calories. Correspondingly mouse 62 kept in the same cage may have eaten more than half of the total amount in the cage. The value of individual caging is exemplified by the results of this experiment. There was some overlapping in the state of epiphyseal development of the mice fed the high-fat diet in restricted quantities and of those fed the high-carbohydrate diet ad libitum. In the first set, mouse 40 (high-carbohydrate diet ad libitum) showed a slightly more youthful growth zone than those fed restricted amounts of the high fat diet (mice 34, 35). However, the exact caloric intake of mouse 40 is not available. Since the final weight of this mouse (26.8 gm.) was lower than that of mice 34 and 35 fed the restricted amounts of the high-fat diet (30.0 and 29.7 gm.), some degree of malnutrition cannot be ruled out as a factor in this discrepancy. On the other hand, the epiphyses of the remaining four mice fed the high-carbohydrate

TABLE 5

WEIGHTS, CALORIC INTAKE, AND MICROSCOPIC FINDINGS IN THE GROWTH ZONES OF MICE

Age: 6 months

Type of diet	Mouse No.	Initial weight gm.	Maximum weight gm.	Total caloric intake	Width of growth zone (μ)	Epiphyseal cartilage: No. of cells columnar: hypertrophic	Epiphyseal cartilage: Hyalinization	Epiphyseal cartilage: Plug formation	Epiphyseal cartilage: Subchondral bony plate	Microscopic age order within each set
High-Prot. ad lib.	E33	9.1	28.3	1649	95	4-6:0-1	+	+	+	1
High-Fat restr.	E34	8.5	30.0	1401[a]	70 irreg.	2-5:0	marked	marked	marked	6**
High-Fat restr.	E35	8.0	29.7	1401[a]	65 irreg.	3-6:0	marked	marked	+	5**
High-Fat ad lib.	E36	10.7	27.8	1512	70 irreg.	2-6:0	marked	marked	marked	7**
High-Fat ad lib.	E37	9.5	30.0	1577	60 irreg.	1-5:0	marked	marked	marked	8
High-Carboh. restr.	E38	8.6	24.3	1648[a]	90	4-7:0	marked	+	+	3
High-Carboh. restr.	E39	8.3	24.4	1648[a]	95	3-7:0	marked	+	+	2
High-Carboh. ad lib.	E40	12.0	26.8	[b]	90 irreg.	5-7:0	+	+	+	4**
High-Prot. ad lib.	E42	14.3	27.6	1570[a]	95	4-6:0-1	+	+	+	2
High-Prot. ad lib.	E43	12.3	24.8	1570[a]	100	4-7:0-1	+	+	marked	1
High-Fat restr.	E44	14.0	24.7	1419	80 irreg.	3-6:0	marked	+	+	5*
High-Fat ad lib.	E45	10.4	29.9	1666[a]	60 irreg.	1-5:0	marked	+	marked	8
High-Fat ad lib.	E46	9.9	31.7	1666[a]	55 irreg.	1-4:0	marked	marked	marked	9
High-Carboh. restr.	E47	14.9	33.6	1473[a]	85	3-6:0-1	marked	+	marked	3
High-Carboh. restr.	E48	15.3	29.0	1473[a]	90 irreg.	4-7:0	marked	+	marked	4
High-Carboh. ad lib.	E49	15.5	31.5	1709[a]	85	3-5:0	marked	marked	marked	6
High-Carboh. ad lib.	E50	14.5	28.7	1709[a]	75 irreg.	4-7:0	marked	+	marked	7
High-Prot. ad lib.	E56	15.2	23.7	1430	110	2-6:0-1	+	+	marked	1
High-Fat restr.	E57	15.5	28.9	1387[a]	65 irreg.	3-5:0	marked	+	+	5*
Hight-Fat restr.	E58	15.5	25.3	1387[a]	60 irreg.	2-6:0	+	marked	marked	6
High-Fat ad lib.	E59	15.4	25.7	1518	55 irreg.	2-5:0	marked	marked	marked	8**
High-Carboh. restr.	E60	17.0	25.1	1347[a]	65	2-6:0	marked	+	+	4*
High-Carboh. restr.	E61	15.5	28.9	1347[a]	70	2-7:0	marked	+	+	3
High-Carboh. ad lib.	E62	17.3	28.5	1351[a]	65 irreg.	1-5:0	marked	marked	marked	7
High-Carboh. ad lib.	E63	17.1	30.4	1351[a]	80 irreg.	3-7:0	marked	marked	marked	2

* Articular cartilage shows slight age changes (grade I).

** Articular cartilage shows moderately advanced age changes (grade II).

[a] Means of total consumption of two animals kept together in one cage.

[b] Not available.

diet ad libitum (49, 50, 62, 63) appeared younger than those of mice 36, 37, 45, 46, and 59 which had been fed the high-fat diet ad libitum.

In the first set, mice 36 and 37 had consumed 1512 or 1577 calories respectively of the high-fat diet and had thus a lower caloric intake than the animals 38 and 39 which had eaten 1648 calories of the high-carbohydrate diet. Yet, the former disclosed a more advanced degree of skeletal development than the latter. On the other hand in animals 36 and 37, epiphyseal development was farther advanced than in mice 34 and 35 which had actually consumed fewer calories of the same ration (1401 as compared to 1512 and 1577 calories). On the whole, the differences between the animals of this age group were slight. Possibly once a certain stage has been reached, there is no marked further response by the degenerating cartilage to dietary influences.

Articular age changes were absent in the mice fed the high-protein diet. Slight articular age changes were noted in mice 44 and 57 which consumed 1419 or 1387 calories of the high-fat diet respectively, and in mouse 60 which had eaten 1347 calories of the high-carbohydrate diet. Moderately advanced articular age changes were seen in mice 34, 35, 36, and 59 which had consumed 1401, 1512, or 1518 calories of the high-rat diet respectively, and in mouse 40 which had eaten an undetermined amount of the high-carbohydrate diet.

Discussion

In individual groups of mice fed diets differing in protein, fat, or carbohydrate contents respectively, skeletal development and ageing proceeded most slowly in the animals consuming a high-protein ration ad libitum, and usually most rapidly in mice kept on a high-fat diet ad libitum, while most mice fed a high-carbohydrate diet ad libitum took an intermediate position regarding the progress of skeletal development. Animals receiving the high-carbohydrate and high-fat rations in amounts restricted to the caloric intake of the protein-fed pilot animal varied somewhat in their relative rates of epiphyseal development. As a rule, the animal kept on the restricted carbohydrate regimen was retarded as compared to that receiving the restricted high-fat diet; in the latter animals, the rate of epiphyseal development showed some overlapping with that of those mice that had consumed a high-carbohydrate diet ad libitum. In some mice fed the latter diet, epiphyseal development approached or even exceeded that

seen in animals fed the high-fat diet in restricted quantities. In some instances, these deviations could be accounted for, in others they could not, and one has to assume that they were due to individual differences in the rate of development and food utilization.

In order to overcome the difficulties encountered in the study of the individual series and to evaluate the significance of the variations observed, an attempt was made to further quantitate the data obtained. For this purpose, all animals of the same age groups fed the high-protein diet ad libitum, the high-fat diet ad libitum or in restricted amounts, and the high-carbohydrate diet ad libitum or in restricted quantities were grouped together, and each animal was given a position according to the microscopic age of the growth zones within the entire age group. Subsequently, the numbers of the position within each dietary group were totaled, and the means were determined by dividing the total by the number of individuals observed in each experimental group. These means in turn were given a rank in ascending order (Roman numerals) according to their numerical values. The figures thus arrived at indicate the position of the dietary group within the particular experimental age group (Table 6).

During all experimental stages, the groups of mice consuming the high-protein diet took rank I indicating that these animals as a group had the youngest looking growth zones. All mice fed the same amount of calories or less but in the form of carbohydrate or fat ranked higher, that is, their skeleton was farther developed than that of the mice kept on the high-protein ration. Of the former diets, the high-fat ration invariably placed the mice in a more advanced stage of skeletal development (rank III was taken in 3, and rank III-IV was taken in one of four stages) than did the high-carbohydrate diet (rank II was taken in 3 and rank III was taken in one of four stages). Thus, the composition of the diet, that is, its contents of fat or carbohydrate respectively, influenced the rate of epiphyseal development.

However, the caloric intake played an additional role in determining skeletal development. This is indicated by a number of facts: In the first age group (2 month-old mice), epiphyseal development of the mice kept on the restricted high-fat diet and of those fed the high-carbohydrate diet ad libitum ranked so closely together that the differences were almost negligible. The mean caloric intake of these two groups was 265 calories for the high-fat restricted, and 339 calories for the high-carbohydrate ad libitum fed series. The higher caloric intake in

TABLE 6

SUMMARY OF MICROSCOPIC FINDINGS IN THE INDIVIDUAL SETS OF ANIMALS

	After 1 month of observation					*After 2 months of observation*				
Type of diet	Mouse No.	Mean caloric intake	Individual ranking	Mean ranking	Group ranking	Mouse No.	Mean caloric intake	Individual ranking	Mean ranking	Group ranking
High-Protein ad libitum	E 9	306	7	$3\frac{2}{3}$	I	E21	579	4	$2\frac{1}{3}$	I
	E14		3			E51		1		
	E64		1			E60		2		
High-Fat restricted	E10	265	10	8	III-IV	E22	531	14	11	IV-V
	E15		9			E52		8		
	E65		5			E70		11		
High-Fat ad libitum	E11	323	15	$13\frac{1}{3}$	V	E23	675	15	$10\frac{2}{3}$	IV-V
	E16		13			E53		5		
	E66		12			E71		12		
High-Carbo-hydrate restricted	E12	288	11	$6\frac{1}{3}$	II	E24	530	13	$8\frac{1}{3}$	II-III
	E17		6			E54		3		
	E67		2			E72		9		
High-Carbo-hydrate ad libitum	E13	339	4	$8\frac{2}{3}$	III-IV	E25	672	6	$7\frac{2}{3}$	II-III
	E18		8			E55		7		
	E68		14			E73		10		

TABLE 6 (*continued*)

	After 3 months of observation					*After 5 months of observation*				
Type of diet	Mouse No.	Mean caloric intake	Individual ranking	Mean ranking	Group ranking	Mouse No.	Mean caloric intake	Individual ranking	Mean ranking	Group ranking
High-Protein ad libitum	E30	877	1	3	I	E33	1555	4	2½	I
	E74		3			E42		3		
	E81		5			E43		2		
						E56		1		
High-Fat restricted	E27	752	13	8⅓	III	E34	1399	17	14	III
	E75		8			E35		18		
	E82		4			E44		16		
						E57		7		
						E58		12		
High-Fat ad libitum	E28	1000	14	13⅔	V	E36	1588	19	17⅓	IV-V
	E76		12			E37		21		
	E83		15			E45		9		
						E46		25		
						E59		15		
High-Carbo-hydrate restricted	E29	809	2	5	II	E38	1489	6	11⅙	II
	E77		7			E39		5		
	E84		6			E47		14		
						E48		24		
						E60		10		
						E61		8		
High-Carbo-hydrate ad libitum	E26	1041	9	10	IV	E40	1530	11	17⅓	IV-V
	E78		11			E49		22		
	E85		10			E50		20		
						E62		23		
						E63		13		

the latter group thus exerted an effect similar to that of the high-fat diet consumed in calorically smaller quantities. Furthermore, in most instances epiphyseal development was farther advanced in animals consuming ad libitum quantities than in those consuming the same diet in restricted amounts. In the oldest age group (6 month-old mice), the animals consuming the high-fat diet and those fed the high-carbohydrate diet ad libitum were identical in rank taking the last place or that next to last. The mean caloric intake of these two groups was likewise similar: It was 1588 calories for the mice kept on the high-fat diet and 1530 calories for those on the high-carbohydrate regimen. The difference in the caloric intake of these animals was small, particularly in view of the prolonged period of observation. The similarity of the microscopic findings may be due to decreased responsiveness of the cartilage: Once a certain degree of regression has been reached, further stimulation will not cause further tissue response. In the mice fed the high-fat ration, this limit might have been reached before the age of six months, while in the meantime the mice on the high-carbohydrate regimen had time to catch up with them.

In the groups observed for two months, the mice fed restricted amounts of the high-fat or high-carbohydrate diets respectively ranked equal with those fed the respective diets ad libitum. This is the only stage at which an overlapping of this kind occurred, and it might constitute a mere coincidence. On the other hand, the fact that it was observed with both diets might indicate that it is more than coincidental. The role of age in the response to various synthetic diets has been stressed by a number of investigators (1). Perhaps at the age under discussion, the caloric intake is of greater significance than at other ages.

In summarizing the results one may schematize even further than was done in Table 6, by determining the order of physiologic age of the growth zones of all mice kept on a certain dietary regimen irrespective of the duration of the experiment. If the rank numbers (Roman numerals) for each dietary group were totaled and divided by four (the number of experimental stages), the following means were obtained:

High-Protein Diet:	1
High-Fat Diet Restricted:	3.5
High-Fat Diet ad libitum:	4.8

High-Carbohydrate Diet Restricted:	2.1
High-Carbohydrate Diet ad libitum:	3.1

Or, expressed in order of physiologic age:

I: High-Protein Diet ad libitum.
II: High-Carbohydrate Diet Restricted.
III: High-Carbohydrate Diet ad libitum.
IV: High-Fat Diet Restricted.
V: High-Fat Diet ad libitum.

While these results appear clear cut, it should be borne in mind that they were arrived at by means of schematization of microscopic findings. It is, however, impossible to express tissue changes in quantitative terms alone. Some allowance has to be made for changes that do not lend themselves to quantitative analysis. The pros and cons of the pair-feeding method have been fully discussed (1). In spite of all precautions, technical pitfalls are unavoidable, and finally computing averages from small samples carries certain objections. Still, the present observations seem to permit conclusions at least concerning trends. Confirmation of these conclusions was obtained in similar investigations (unpublished data) in which the various diets were fed for periods of several months; at the end of these periods, the animals were put on an ad libitum regimen of Purina Laboratory Chow. The microscopic differences in epiphyseal development were the same as those observed in animals fed the diets to the end of the experimental periods. These observations demonstrate in particular the irreversibility of the changes induced; moreover, they indicate that these changes were not the result of any deficiencies in the diets fed (2, 3, 6).

Summary

In male mice of strain C57 black fed ad libitum diets varying in protein, fat, or carbohydrate contents respectively, epiphyseal development and ageing proceeded most slowly in the animals fed the high-protein diet and most rapidly in those receiving the high-fat ration with those kept on the high-carbohydrate regimen taking an intermediate position. Of the animals fed isocaloric amounts of high-protein, high-fat, or high-carbohydrate diets respectively, those on the high-protein ration likewise showed the slowest and those on the high-fat regimen the fastest rate of skeletal development and ageing, while those receiving the high-carbohydrate diet disclosed a rate of epi-

physeal growth intermediate between that observed under the influence of the other two diets. Ad libitum fed animals consuming larger quantities of the same diet than those on the restricted rations showed more advanced skeletal development than the latter. It is, therefore, concluded that the amount of calories consumed as well as the fat contents of the diet specifically influence skeletal growth, development and ageing.

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